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THE BEHAVIOR OF ISOLATED PIECES OF ASCIDIAN (PEROPHORA VIRIDIS) STOLON AS COMPARED WITH ORDINARY BUDDING[1,2]

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Plates 15–17

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[2] The experimental part of this work was carried on in the Biological Laboratory of the United States Bureau of Fisheries at Beaufort, North Carolina, during the summers of 1930, 1931, and 1932. Thanks are expressed to the Commissioner of Fisheries for the privilege of admission to the laboratory, also to the two consecutive directors, Dr. S. F. Hildebrand and Dr. H. F. Prytherch, and members of the laboratory staff for their cordial assistance. Sincerest gratitude is expressed to Dr. H. V. Wilson, under whose direction the work was carried on, for his helpful constructive criticism.

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INTRODUCTION

The gross structure and general growth habits of the social ascidians have been described by Giard (8), Kowalevsky (10, 11), Ritter (14, 15), Lefevre (12, 13), Brien and Brien-Gavage (2, 3, 4), and many others. *Perophora viridis*, the subject of this investigation, forms colonies of greenish zooids connected by tubular, branching, green stolons which creep over wharf pilings, hydroids, bryozoa, or the tests of other and larger tunicates (fig. 1).

In order to obtain data for comparison of the two phenomena, i.e., the normal budding method carried on in the establishment of a colony, and the transformation of small pieces of the stolon into zooids, the following technique was employed.

Stolons of freshly collected Perophora, growing on and among hydroid and bryozoan colonies, were cut into one and two inch lengths and tied on new, clean, glass slides. The slides were then suspended near the top of a battery jar of sea water which was dipped from the surface at rising tide; the water in the jars was changed twice daily. Placing the jars in an aquarium table of running salt water kept the temperature fairly constant. Within forty-eight hours after the material was tied on the slides there were new, healthy, green stolons growing out beyond the original cut ends (fig. 1).

Pieces of these new stolons ranging in length from one-half to two millimeters were abscised and placed on slides in finger bowls of filtered sea water which was also dipped from the surface at rising tide. This water was also changed twice daily. The small stolon-pieces were observed and sketched every twelve hours. Transforming pieces of stolon were preserved in Bouin without acetic, in normal Bouin, and in Allen's modification of Bouin. The pieces were stained *in toto* in haemalum, imbedded, sectioned 5μ, and counter-stained in either acid fuchsin or methylen blue. Freshly collected Perophora material showing blastozooids of all ages were treated in the same way and a comparative study was made.

EARLIER PHASES OF DEVELOPMENT

a. Establishment of a young colony by the Oozooid

The normal method of origin of a young colony of *Perophora viridis* was observed under controlled conditions in the laboratory during the course of the investigation. Young oozooids, freshly hatched and about 1–1.3 mm. in length, were removed from the cloacal cavity of the parent zooid, placed on slides in finger bowls of filtered sea water and observed at frequent intervals. The actual time of, and the various steps in the metamorphosis of the young oozooid, or tadpole, are given:

6/17/32, 2:30 p. m., young oozooids were removed from the cloacal cavities of adults which were freshly collected from the harbor. The tadpoles appeared fully formed, but were still inclosed in the egg membrane. 3:00 p. m., tadpoles had become free-swimming and were moving about with a sort of sculling movement of the tail. 5:00 p. m., metamorphosis had begun; the sensory vesicle had decreased in size, and the larval sense organs had disintegrated; the notochord was an amorphous mass at the base of the tail; the adhesive papillae had elongated; the heart was observed beating, and streams of blood could be seen passing in and out of the two ends of the heart. 6/18, 9:00 a. m., metamorphosis was complete: branchial stigmata and siphons were present. There was no sign of either a tail or a notochord. Only a small remnant of the sensory vesicle remained—between the siphons. The outgrowths from the adhesive papillae had become definitive stolons, four in number. Two of these stolons were green in color and were beginning to attach to the slides on the bottom of the dish. 6/22, only two of the original four stolonic out-growths seemed to be flourishing. 6/28, only one healthy stolon remained. This bore a young bud or blastozooid about 2 mm. from the oozooid.

Except for some details the metamorphosis from tadpole to adult oozooid is well known. When the first bud has been formed successively younger ones appear nearer the tip as it grows forwards on the substratum. The growing stolons give rise to side branches which also bear buds; thus are formed complex colonies in which it is soon impossible to distinguish the original oozooid and its original stolonic outgrowths.

This account of the establishment and the morphology of a colony agrees with that of Brien and Brien-Gavage (4) for *Perophora listeri*, but differs very strikingly in respect to morphology from a species of the Pacific coast, *Perophora annectens*, as described by Ritter (14). The

latter says that the Pacific coast species may be incrusting, i.e., very flattened, and that the individuals and stolons may be enveloped in a common tunic. In the possession of simple linear stolons on which are borne the blastozooids of varying ages and sizes, *Perophora viridis* and *P. listeri* both differ in morphology from *Clavelina lepadiformis* as described by Brien and Brien-Gavage (3). "Clavelina sends out from the base of the post-abdomen radial stolons which are solidly fixed to the support. Each stolon branch generally presents at its distal end a dilatation which soon detaches (from the ascidian) and becomes the blastogenetic center at the expense of which a blastozooid is formed. Blastozooids of the same cluster have no direct or physiological connection with each other. They do not form true colonies."

b. General structure of the normal stolon

The structure of the stolon is not so simple and definite as some of the earlier accounts describe it. The wall of this tubular structure really consists of three layers of tissue: (1) an outside tunic with scattered cells and much intercellular substance, (2) a thin epithelio-ectodermal layer in which cell boundaries are, with the technique employed, not visible in sections, and (3) a delicate layer of mesenchyme cells, interconnecting often, forming a discontinuous lining of the stolonic cavity. This cavity is divided vertically by a thin single-layered septum attached at both edges to the ectodermal layer of the wall and continuous with bits of the mesenchymal lining which chance to lie along the line of attachment (fig. 2). Right and left halves of the stolonic cavity communicate at the distal tip, as the septum is lacking for a distance of one-fourth to one-half millimeter at this point. The origin of the septum of *Perophora viridis* was not ascertained in this study, but Brien and Brien-Gavage (3, 4) state that it is mesenchymatous in origin both in *Clavelina lepadiformis* and in *Perophora listeri*. They describe how it is formed by the fusion of the wall of two blood sinuses growing out from the body of the oozooid. The connections between the blastozooid and the stolon are found here, as in Brien's investigation, quite like those existing between the oozooid and the stolon. A discussion of this point is given later.

c. Cellular components of the stolon

Sections were prepared with the technique mentioned above and the living stolon unstained and after staining with weak (1:10,000) neutral

red was repeatedly studied. Examination with the immersion objective and a reasonably high ocular is necessary.

Observations on the living stolon. In the living stolon stained with neutral red the cell most in evidence in the tunic was a large amoeboid body containing six to eight dancing, red granules (fig. 3). This is undoubtedly the compartmental amoeboid cell described by George (6) although the "compartments" could not be seen with the technique employed (George employed for this purpose another technique). These cells are listed among the free blood cells of the haemocoele by George, but in this investigation they could not be observed in the haemocoele of the living unbroken stolon. However, a living stolon whose ectoderm and tunic were punctured before being stained with weak neutral red, showed these cells with the red granules among the blood cells which streamed out of the punctured place.

In an instance or two some large, pale amoeboid cells were observed creeping into the tunic from the haemocoele through the ectodermal cells on the distal end of the stolon. These were probably of the compartmental amoeboid type, but since the specimen under observation at the time was not stained no granules were to be seen. Possibly these cells become tunic cells. If so, the observation in a measure confirms Brien's conclusions as to the origin of the tunic cells. Brien (2) says that, in a detunicated piece of Clavelina stolon, the presence of ectoderm is necessary to the reformation of the tunic. However, he believes that the actual tunic substance may be borne by migrating, vacuolated, mesenchymal cells which wander out of the haemocoele through the ectoderm and they, upon contact with water, yield the substance which forms the new tunic.

In the living stolon the ectoderm at the tip is seen to be thick (fig. 3); elsewhere it is thin. The boundaries of tall columnar cells are sometimes faintly visible where it is thick. Within the haemocoele or stolonic cavity the septum may be seen as a thin, vertical plate in the median line. The free, circulating blood cells which are easily seen in the haemocoele of the unbroken stolon are, in the terminology of George, green cells, morula cells, signet-ring cells, and orange cells, and some pale colorless types which are presumably lymphocytes. The nuclei are not visible in any of these living circulating cells. A green cell and a morula cell are shown in figs. 6a and 7a respectively. There is no need to describe these cells in the living state as this has been done in admirable manner by George (6, 7), but it is necessary to identify the several types in the fixed material and every effort has been made to do this.

Observations on fixed material. Not all the cells of the sectioned, stained material were easily identified with types of cells of the living stolon. In the tunic of the fixed material the most abundant cell is an irregularly amoeboid body with a fair sized reticulate nucleus which is more often elongated than spherical in form. The range in size of these cells is: length, 7–13μ; width, 3–6μ; nucleus, 3–4 x 2–2$\frac{1}{2}\mu$, or if spherical, 3–3$\frac{1}{2}\mu$ in diameter (fig. 4, b & c). These sizes are close to those of the tunic cells with dancing granules seen in the living stolon. Often several of these cells are seen to be connected by cytoplasmic strands, or they may be isolated, in which case they may or may not show pseudopodial protrusions of their cytoplasm. Very rarely they form a layer, almost epithelial, at the inner wall of the tunic next to the ectoderm. These cells of the fixed material are doubtless the cells with dancing granules, that is, the compartmental amoeboid cells of George (6). Proof lies, however, only in their abundance, size, and position since the granules are not seen in the preserved cell nor the nucleus in the living cell. The compartments are noticeable in the fixed material only in rare cases. George states that a special method of treatment is necessary to bring out this feature in the living cell. Absence of the granules in the preserved form is probably due to their dissolution by the fixative. Bodies which possibly correspond to these granules are recorded by Brien (2) as present in the cells which pass out of the haemocoele to give rise to the tunic. Brien was able to preserve these inclusions by the use of Bouin without acetic acid. This method failed to preserve the granules in the tunic cells of Perophora.

Another type of cell found in the tunic of the fixed material is a rather smooth amoeboid form containing a deeply staining homogeneous body which is probably the nucleus (degenerating?) since no other nucleus is to be seen (fig. 5a, b, c).

In the fixed, stained material, the ectodermal cells at the distal end of the stolon clearly show their columnar structure; elsewhere they are flat and thin. Cell boundaries are never very distinct in the ectoderm at any point. The oval nuclei of the ectodermal cells are poor in chromatin, most of which is usually confined to a nucleolus and perhaps a scattered particle or two. Very rarely there are two small chromatin masses of approximately equal size. A distinct nuclear network cannot be made out.

The septal cells are exceedingly thin and flat in the non-budding region, less so in the budding region. No cell boundaries are visible in the septum at any time, either in sectional view (fig. 2) or in face view.

The oval nuclei resemble the nuclei of the ectodermal cells in the paucity and arrangement of the chromatin. In their staining reactions, also, the ectodermal and septal cells are very similar.

In both the preserved and living material there are three types of free blood cells in the haemocoele which are easily identified with George's cells. These are the green cells, morula cells, and signet-ring cells. The abundant green cells present practically the same appearance in both the living and the fixed stained material, at any rate they retain the green coloring and vacuolated appearance. The nucleus in the fixed cells may be seen as a slightly eccentric, reticulate, vesicular body lying in a mass of homogeneous cytoplasm from which radiate cytoplasmic strands between the several vacuoles of the cell (fig. 6b, c). The cells measure from 7 to 10μ in diameter.

The morula cells in their appearance suggest early stages of segmenting eggs. Their subdivisions rarely exceed 4 to 6 in number. Single, small, reticulate, oval nuclei may occasionally be seen in material which has been destained sufficiently (fig. 7b & c). The range in size of these cells is about 6 x 11μ to 9 x 12μ.

Only an occasional signet-ring cell is found. These cells have a size range about like that of the green cells. There is a fair sized reticulate nucleus in the thicker part of the rim of cytoplasm which surrounds the large eccentrically placed vacuole (fig. 8). The orange cells of the living stolon are not identifiable in the sectioned material.

One very abundant type of spherical cell which stains a brilliant cerise with acid fuchsin can probably be identified with George's granular amoebocyte. This cell is packed with spherules which seem to vary in density and which very often obscure the small solidly-staining nucleus. The diameter of this cell is always around 9μ and that of its nucleus from 2½ to 3μ. Under certain physiological conditions this cell stains a mulberry red instead of cerise, but it is always easy to identify by its spherules (or granules) and by its constant size and form (fig. 9).

One type of free cell in the haemocoele closely resembles the most common tunic cell. Its cytoplasm is apparently homogeneous and it usually stains very lightly. The nucleus is reticulate and of the same average size as that of the tunic cell which has been designated the compartmental amoeboid cell. This cell is usually a bit more rounded in form when found in the haemocoele (fig. 4a). It may occasionally be seen making cytoplasmic connections with others of its kind and even forming small syncytia. Very often it is found closely applied to the ectoderm through which it might easily migrate.

Finally, for this investigation, the most interesting and important type of cell in the haemocoele is a small lymphocyte with a scant amount of finely granular cytoplasm surrounding a relatively large nucleolate nucleus (fig. 10 a–d). These cells measure from 5 to 8μ in diameter. When rounded in form their average diameter is 6μ. The nuclei measure 3 to 6μ in diameter, the average size being 4μ. These cells are almost always spherical although one is occasionally seen putting out a blunt cytoplasmic process. In addition to the nucleolus there are often scattered bits of chromatin in the nucleus, more of them than are to be found in the nuclei of the ectodermal and septal cells. Rarely there is found a lymphocyte in which instead of a nucleolus there are scattered chromatin particles looking as if they might be parts of a reticulum or nuclear network (fig. 10d). In their most simplified form (fig. 10a) these cells look very much like the figures of the lymphocyte type described by Brien and Brien-Gavage (4) for *Perophora listeri*, and again like those of a Bermuda ascidian, *Symplegma viride*, described by George (7). On the contrary George's description of the lymphocyte of *Perophora viridis* shows it to be more like the type in figure 10d which may possibly be regarded as an early transition stage between the primitive lymphocyte and some one of the more specialized forms, e.g., the tunic cell.

Cells resembling lymphocytes may often be seen applied to the septum, or to the ectoderm (fig. 2). Similar cells with processes (fig. 11a, b, c) often interconnecting with one another occur in the haemocoele. These are presumably homologous with the mesenchymal cells of Brien and Brien-Gavage (3, 4) which they say line the entire haemocoele in *Clavelina lepadiformis* and *Perophora listeri*. In *P. viridis* they do not make a continuous lining, but form a discontinuous layer just inside the ectoderm, and they occasionally are found free in the haemocoele as if in migration. Brien (2) states that these and the lymphocytes are different stages of the same element, a conclusion which appears probable.

The above account of the normal structure of the stolon of *Perophora viridis* differs in several details from Lefevre's (13) description. He considers the entire septum as a "collapsed cylinder the walls of which are pressed closely together and attached along the upper and lower borders to the inner surface of the ectodermal tube." Lefevre does not distinguish between the different types of free blood cells. Otherwise his account is correct. Kowalevsky (10) likewise describes the septum of *P. listeri* as being formed of two layers of cells. On the

contrary, Brien and Brien-Gavage (4) state that in *P. listeri* the septum is single everywhere except in actively budding regions. This is the condition in *P. viridis*, and the point is an important one in morphogeny. As to the method and place of origin of the septum in *P. viridis*, this has never been definitely worked out. Lefevre (13) threw doubt on its endodermic, epicardiac origin, a view held before his time from the general likeness of this species to the Clavelinas. Van Beneden and Julin (19) had previously stated that the septum of the stolon of Clavelina had its origin in the epicardium, an outgrowth from the median wall of the pharynx above the heart. Lefevre found no epicardium in Perophora, but was unable to get oozooids of Perophora to transform sufficiently to see the origin of the septum. Brien and Brien-Gavage (3, 4) have found the septa of stolons of both *Clavelina lepadiformis* and *Perophora listeri* to have a mesenchymatous origin, to which account reference has already been made.

In their classification of free mesenchymatous or blood cell types of *P. listeri*, Brien and Brien-Gavage (4) have described homologues of certain of the above blood cells of *P. viridis* as described by George (6, 7), viz., green cells, signet-ring cells, orange cells, and lymphocytes, but they have not been able to establish identity between all the blood cells of the two forms. The three authors, however, agree that the lymphocyte is the most primitive type of blood cell and that transition stages between this and the more specialized types can be found. Brien and Brien-Gavage (4) have figured such transition forms between the lymphocyte type and all the more specialized blood cells of *Perophora listeri*. This has not been done for *P. viridis*, although George (7) has found in another genus, Ecteinascidia, what he believes to be intermediate stages in the formation of some of the highly specialized types of blood cells.

NORMAL BUDDING

New young buds or blastozooids, of the Perophora colony, are almost invariably formed between the smallest visible one already formed and the growing tip of the stolon. Many stolon tips with incipient budding stages were examined, first *in toto*, then as serial sections. No differences of any importance were observed, in the normal budding process, between material freshly collected from the outside and that grown on slides in the laboratory as described above.

For convenience of description the budding process may be divided

into three stages: (1) the formation of a small vesicle from the upper part of the septum, a stage not usually visible externally; (2) the growth of this vesicle and the accompanying formation of certain organ primordia, this stage marked by a hemispherical protrusion of the upper surface of the stolon; (3) the growth and differentiation of the organ primordia. The last stage is visibly accompanied by a great increase in size of the bud, by its constriction from the stolon, by its inclination toward the stolon tip, and, finally, by the assumption of activities such as heart beat, contraction and expansion of the siphons. Naturally, hard and fast lines cannot be drawn between these stages.

First stage in development of bud. The following description of the first stage is based on the study of a series of transverse sections of a stolon which was collected and preserved immediately in Allen's modification of Bouin.

The tunic varies in thickness. It may be very thin. At the distal end of the stolon it is about 21μ thick. The ectoderm at this end is columnar, as said, and about 10μ thick. In both cross and longitudinal sections the cells appear to be more or less loosely joined together, i.e., they are marked off from one another by vague light streaks. Such an arrangement would be well suited for the migration of cells from haemocoele into tunic. Back of the tip the ectodermal cells become thinner and finally reach their attenuated form within a distance of about 100μ. For a distance of about 200μ the ectodermal wall incloses an undivided cavity, the haemocoele, circular in cross section, whose only contents are a few scattered blood cells; there is no sign of a septum.

At a point 230μ back of the stolon tip the septum appears, in the midst of the free blood cells of the haemocoele, as an irregularly polygonal mass of cells representing the end of a rod or plug (fig. 13). Within the distance of 14μ this rod becomes a one-layered plate of cells which soon (15μ) becomes two-layered in its upper part (fig. 14). It is noticeable and remarkable that no cell boundaries are visible in this mass, and yet, with the technique employed, one would expect them to be visible if there. Immediately (7μ) back of this point, the two-layered upper part of the septum acquires a cavity, while its undivided lower part shows, in section, two rows of nuclei in the thickened cytoplasm (fig. 15). Behind this point the cavity extends ventrally through the greater part of the septum, until, at a distance of 70μ from the beginning of the cavity, the section shows an egg-shaped vesicle 40μ wide and 64μ high. The larger part of the vesicle is directed toward the upper, unattached side of the stolon, while its lower, smaller part joins the small

remnant of the stolonial septum here consisting of a single layer of cells (fig. 16). Proximally, that is, toward the ascidiozoöid from which the stolon grew out, the vesicle which is the inner or endoblastic vesicle of a young bud, gradually decreases in size, until, at a distance of 200μ from its origin it comes to an end as a vesicle. At this point it passes into the septum which here has the same form as it has just distal to the vesicle, i.e., thickened above and one-layered below (fig. 14). Thirty-five micra proximal to the vesicle the whole septum becomes a one-layered plate of cells which gradually (in a distance of 308μ) becomes extended entirely across the haemocoele. This condition persists for the distance of 1 mm. At the end of this distance a well developed bud is found attached to the stolon.

The above description is based, as said, on the study of a stolon collected in the harbor. Similar studies were made of stolons grown on glass in the laboratory. There were no differences of interest. To the description as given certain minor additions may be made, some observed in stolons grown in nature, some on those grown in the laboratory. In the first place the nuclei of the blastogenetic part of the septum (figs. 13–16) are characteristic septal nuclei, i.e., they are oval and nucleolate, although slightly larger than nuclei of the septum in a non-budding region. Again the vesicular swelling may at the middle level of the vesicle involve the entire septum (fig. 17a), the remnant of the septum shown in fig. 16 disappearing as it too becomes vesicular. Again there are certain irregular eminences on the outer wall of the young endoblastic vesicle which have some interest and to which return is made later. And also the septum in the immediate neighborhood of an endoblastic vesicle may show certain extensions prolonged out into the haemocoele as in fig. 18. Such eminences and extensions at first sight would be thought of as proliferations of the septal tissue. But there are some facts which lead to the idea that they really represent cell masses of the haemocoele which have united with the septum.

Brien and Brien-Gavage (4) have noted similar projections from the septum of *Perophora listeri*. They regard such spots as places of attachment of free mesenchymal cells of the haemocoele, and say that they furnish further proof of the mesenchymatous origin of the septum.

This question of the participation of free blood cells in the formation of the blastogenetic septum is taken up later.

While the above account of the origin and early appearance of the bud anlage agrees essentially with the accounts of Kowalevsky (10), Lefevre (13), and Brien and Brien-Gavage (4), there are some differences espe-

cially from the findings of the first two investigators. Kowalevsky finds the inner vesicle of the bud arising as a lateral evagination of one of the two layers of a permanently double septum. This, of course, would make the buds appear laterally, i.e., to left or right of the septum, instead of dorsally on the stolon, and his figures show this lateral location. Lefevre, also, considers the septum as everywhere and permanently two-layered, and of a very constant and regular nature, which is obviously not the case. He, however, shows the bud protruding dorsally from the stolon—the correct position. Brien and Brien-Gavage agree with Lefevre except in the matter of the structure of the septum. This they find to be of mesenchymatous origin, and, except in the budding region, one-layered.

In respect to the structure of the septum the results of this investigation are in general agreement with those of Brien and Brien-Gavage (4). The idea held by Kowalevsky and Lefevre that the septum is a two-layered sac was in all probability based on the study of blastogenetic parts of the septum. Sections of the septum in such places, e.g., fig. 17a, may be quite like the sections figured, especially by Lefevre (13).

The growth changes involved in the formation of the inner vesicle of a young bud are of interest.

(1) As has been said, the septum in its apical region (figs. 13, 14) does not extend from ectoderm to ectoderm across the haemocoele as it does elsewhere. This is to be expected since we are dealing with a growing end. The free end is to be looked on as the earlier state. With nuclear proliferation and growth the plate-like state is reached.

(2) Cell multiplication undoubtedly occurs in the septum of a budding region, since mitotic figures may be seen. But while the growth in such a region is active, the mitotic figures are few. There are some suggestions that amitosis occurs. At any rate many nuclei are observed which contain two distinct masses of chromatin of equal size and often such nuclei have a constriction in the middle region.

(3) Finally, there are many indications that lymphocytes and the mesenchymal cells, scarcely to be distinguished from them, are added to the septum. Thus in fig. 19 are to be seen two such cells in contact with the septum and another cell which has already established cytoplasmic continuity with the septum. And many such instances are found. Moreover, there are to be found little plates of cells (fig. 17b) in the haemocoele in the region of the young bud, but not elsewhere, and in these the nuclei and cytoplasm have the same appearance as in the lymphocytes. The irregular projections already mentioned as

occurring on the septum, still in its plate-like condition (fig. 18), and on the wall of the endoblastic vesicle are presumably to be looked on as formed by the coalescence of such little plates with the septum or vesicle respectively. Thus in the transformation of the plate-like septum into a vesicle, while intrinsic growth, possibly with some amitosis, plays the more important part, it would seem that the amount of available substance is also increased by the incorporation of free lymph cells or of little masses formed by the fusion of such. This view is in general accord with that of Brien and Brien-Gavage (3, 4).

Enlargement of the endoblastic vesicle and formation of a protruding bud. One of the earliest steps in this phase of the development consists in a thickening of the wall of the endoblastic vesicle and of the ectoderm dorsal to it.

As respects the growth of the vesicular wall it is found that mitoses are especially numerous in it at this stage and it may therefore be concluded that intrinsic growth is going on. Also there are microscopic pictures which indicate that, as at an earlier stage, lymphocytes continue to fuse with it. As to the dorsal thickening of ectoderm, mitotic figures are not found in this region, but again pictures which look like stages in amitosis occur with frequency.

Following on this increase in thickness of the vesicular wall in general and of the ectoderm dorsal to it comes the stage usually referred to as the protruding bud. Among the growth processes involved in this change, it may be noticed that the localized thickening of ectoderm has disappeared. It may possibly be looked on as a preliminary nutritive state preparatory to the establishment of a greater area of comparatively thin ectoderm. The increase in size of the endoblastic vesicle is doubtless, as said, brought about chiefly by its intrinsic growth. In its protrusion the ventral septal remnant, shown in fig. 16, seems to be a causal factor. Comparison of section figures of the earlier stage (fig. 16) and the stage of the just protruding bud shows that this plate-like remnant thins out and lengthens in a dorso-ventral direction. The effect of this would be to push the endoblastic vesicle dorsally. At this stage there is no special change in the thickness of the vesicular wall. As already said, there are buds in which the entire septum is locally transformed into a vesicle. In such cases the causal factor just alluded to would apparently not exist. Lefevre (13) has discussed this question.

As the young bud grows dorsally, it begins to constrict off from the stolon laterally and to incline toward the free tip of the stolon. In this stage, then, the young bud forms a more or less spherical protrusion on

the dorsal wall of the stolon usually about one and a half to two millimeters from the stolonic tip. In its gross structure such a bud consists of two vesicles, an inner formed by the stolonic septum with which it is still connected, and an outer vesicle formed by the stolonic ectoderm; a tunic layer, continuous with the stolonic tunic, covers the bud. Between the inner and outer vesicles of the bud there is a cavity, the haemocoele which is in direct communication with the haemocoelic cavities of the stolon. Thus free blood cells of the latter cavities have access to the haemocoele of the bud.

Organogenetic processes in the bud. In its organogenetic processes the young bud appears to follow rather closely the method described by Lefevre (13). In brief, Lefevre found that the inner or endoblastic vesicle of the bud formed the peribranchial chambers and cloaca, as well as the pharynx and entire digestive tract, while free blood cells formed the nervous system (dorsal tube and ganglion), pericardium (and heart) and the gonads.

In a bud of about the same age as the one described above there may be found on the dorsal wall of the endoblastic vesicle near its anterior end, i.e., the end directed toward the distal end of the stolon, a small aggregation of lymphocytes. This aggregation, in the cross-sections, first appears in a linear shape, but later takes the appearance of a solid, rounded mass which is applied rather closely to the dorsal wall of the vesicle, i.e., the primordium changes from a loose sheet of cells to a solid cord lying anteriorly in the median line on the dorsal side of the inner vesicle, and lying posteriorly a bit to the left of the median line. From this mass of cells, and possibly other additional lymphocytes which subsequently join it, are formed the hypophyseal organ and the ganglionic mass dorsal to it. At no time have cells from the dorsal wall of the endoblastic vesicle been observed in the act of passing into this mass, although such an action would not be impossible to conceive, since cells of both organs seem to be identical in structure and perhaps in origin.

In buds thus exhibiting the primordium of the nervous system there can be found more posteriorly, near the center of the right side of the endoblastic vesicle, a similar, but perhaps slightly larger, solid aggregate of lymphocytes, the primordium of the pericardium (and heart). This mass subsequently acquires a cavity and the surface turned toward the inner vesicle invaginates to form the heart. As in the case of the anlage of the nervous system there seems to be no evidence that cells enter the pericardial mass from any source other than the blood stream,

although this anlage, too, is rather closely applied against the wall of the inner vesicle after the aggregation has reached considerable size. Both primordia, the posterior end of the nerve-hypophyseal and the anterior end of the pericardium, are shown in the transverse section, fig. 20. The nerve-hypophyseal primordium is again shown at a higher magnification in fig. 21, the pericardial primordium at a higher magnification in fig. 22.

Shortly after the cell aggregates constituting the primordia of the nervous system and pericardium have reached a compact solid form, the middle and posterior regions of the endoblastic vesicle undergo a rotation of about 90 degrees. Lefevre (13), the first accurately to describe this action, says that the rotation is brought about by the thinning of the wall of the vesicle except on the right side. The present investigation verifies Lefevre's findings. The rotation results in bringing the thickened right side of the vesicle to a ventral position, and in shoving the connection of the septum and former ventral wall of the vesicle to the left ventro-lateral region. The thickened region now brought ventrally subsequently becomes the endostyle.

The rotation of the inner vesicle also affects the position of the pericardial and dorsal tube primordia which are at this stage rather closely pressed against the vesicle. The pericardium is now pulled down to a position on the right ventro-lateral region of the vesicle where it remains permanently. The middle region and posterior end of the primordium of the nervous system are brought to a mid-dorsal position. It will be recalled that the anterior end of this anlage forms in approximately the median dorsal line. The position of this portion is not disturbed since the rotation affects the anterior end of the vesicle very little.

Concurrently with the rotation of the inner vesicle there appear in its latero-ventral wall two parallel, longitudinal furrows, one on each side of the future mid-ventral line, which press dorsally and slightly mesially so that their inner walls meet before touching the dorsal wall of the vesicle. Thus the original endoblastic vesicle is divided into an upper and outer part, crescentic in cross section, which becomes cloaca and peribranchial chamber, and a lower median vesicle which becomes the pharynx. As Lefevre (13) points out, at first the two sides of the peribranchial chamber are asymmetric; the right side extends farther anteriorly, while the left side extends farther posteriorly. As development proceeds these differences are eliminated, and the lateral, anterior, and posterior borders of the outer vesicle are extended to surround the pharynx. However, the extreme anterior and posterior ends and ven-

tral side of the latter are never covered entirely by the outer vesicle. The pharyngeal and peripharyngeal cavities are put into communication later by the formation of a series of branchial stigmata.

About the time the peribranchial chambers and cloaca are being separated from the pharynx, there grows out from the left, dorsal, posterior wall of the latter a small tubular evagination. This tube, which is the primordium of the digestive tract, elongates and curves, first ventrally, then anteriorly, and finally dorsally, until it reaches the cloacal wall. In the meantime the tube has differentiated into three regions, (1) a slender sharply curved esophagus, (2) an enlarged stomach, and (3) an intestine which is longer than the other two regions combined and which itself has two enlargements near its origin. Sometime later an opening is formed at the point of contact between the intestine and the cloacal wall. Later, also, there arises from the wall of the stomach a slender evagination, the "organe refringent" which branches many times, each branch finally ends in an enlargement, ampulla, pressed against the intestinal wall.

While these developments have proceeded in the digestive tract, the pharynx and peribranchial chambers have been establishing communication by the formation of branchial stigmata. The latter seem to form according to the method described by Lefevre (13). Thickened spots appear first in the pharyngeal wall. These spots are evaginated slightly to touch the inner wall of the peribranchial chambers and this invokes a thickening of the latter walls at the point of contact. The two walls fuse where contact is made and an opening is formed at the point of fusion. However, the first thickening occasionally appears in the wall of the peribranchial chamber as Beers (1) has described for Ecteinascidia.

While the earliest stigmata are being formed the primordia of the oral and atrial siphons can be seen in the process of formation. These, too, follow the same course as described by Lefevre. Ectodermal invaginations occur, one over the anterior end of the pharynx and one over the middle of the cloaca. Where these touch the pharyngeal and cloacal walls the latter are thickened slightly. Later an opening breaks through at the bottom of each invagination. Around the invaginations for both siphons many mesenchymal cells aggregate. These form the chief muscles of the body, i.e., those which control the siphons.

Connections between stolon and blastozooid change considerably as the latter grows. At first, the young bud lies at right angles to the stolon, and the haemocoelic cavity of the bud opens widely on each side into the corresponding half of the haemocoele of the stolon (fig. 20).

By the rotation of the endoblastic vesicle its connection with the septum is shoved to the left. As the peribranchial furrows are formed the left one is placed mesially to the connection of the septum and the vesicle so that the septum remains in connection with the left peribranchial primordium. The latter connection lasts for a time, but is broken during the further growth of the bud. In this growth the bud becomes inclined toward the tip of the stolon and is moreover more distinctly constricted off from the stolon. As the zooid increases in size and assumes activities, this narrowed connection with the stolon increases in length and assumes the aspect of a stalk. The stalk is finally divided into two at the point of exit from the zooid, and these two, a and b, are shown in transverse section in fig. 24a and b. In the region of connection with the stolon the stalk remains undivided and is shown in transverse section in fig. 25. The stalk divided or undivided, contains, of course, the haemocoele with free blood cells, figs. 24 and 25. At this time some of the haemocoelic cells within the blastozooid unite, as Brien and Brien-Gavage (4) describe, to form the walls of two sinuses. These sinuses they designate subendostylar and subintestinal. The former is directly continuous, as Brien and Brien-Gavage say, with the heart, the latter is said to be indirectly connected with the heart through the perivisceral network of sinuses. The two sinuses are shown in fig. 23 which represents a part of a section through a young blastozooid. These two sinuses are extended into the two divisions of the stalk-like connection between zooid and stolon. In fig. 24b the imperfectly formed wall of one of the sinuses is shown, while in the other division of the stalk, *a*, the sinus formation has not begun. In the undivided part of the stalk the walls of the two sinuses come in contact and partially fuse as is shown in the transverse section in fig. 25. This region of fusion is continuous with the septum of the stolon. The two sinuses are continuous with the two haemocoelic cavities of the stolon, one on each side of the septum. The septum is thus seen to be quite continuous with mesenchymal structures of the blastozooid, and as already stated, Brien and Brien-Gavage (4) find that in the oozooid it originates as a mesenchymal structure.

No mention has yet been made of the gonads. The early common primordium of these is to be seen as a group of mesenchymal cells in the loop of the intestine about the time the latter becomes fully differentiated. The transformation of this primordium into the gonads and their ducts has not been followed. Lefevre (13) has covered this ground.

Only a brief account of organogenesis has been given since the various

steps have been found to agree in nearly all details with Lefevre's description (13). This short account will serve as a basis for comparison between the processes as they occur in normal budding and in the abscised pieces.

ISOLATED PIECES OF STOLON

Early behavior of the longer pieces

The stolons of the Perophora stock-material, which was grown in the laboratory in a previously described manner, appeared normal in structure and budding activities. Hence it was assumed that isolated pieces of such laboratory-cultured stolons would act in essentially the same way as isolated pieces of the natural, freshly collected stolons. The latter would have been much more difficult to isolate. Accordingly, loose, unattached ends of the stolons, which projected conveniently beyond the material originally tied on the slides, were cut off, sectioned into small pieces ranging from one-half to two millimeters in length, and placed in finger bowls of sea water which was carried in from the outside and filtered.

Within one to three hours after being cut, all pieces seemed to be healed at each end and to have shrunken slightly within the tunic which had contracted and covered the cut surfaces. Often, within 24 hours after being cut, the ends of the pieces had extended outside the bounds of the old tunic and had secreted a delicate new tunic layer. Sometimes the entire piece withdrew from the old tunic and secreted a new tunic around itself.

In general it may be said that the longer pieces, i.e., those of more than one millimeter in length, usually elongated slightly and budded in apparently normal fashion. The majority of such buds were placed normally on the stolon piece; however, an occasional case of terminal budding occurred in these pieces. Brien and Brien-Gavage (2, 3) found terminal budding to be the rule and not the exception in isolated bits of Clavelina stolon. Some of the longer pieces of Perophora stolon showed signs of budding very shortly after isolation and before any noticeable elongation had taken place. These pieces probably came from incipient budding regions which had their activities only temporarily interrupted by cutting. Examination of sections showed, in practically all cases, that the isolated stolon-piece had modified the budding process slightly, no matter how little time elapsed between isolation and appearance of the young bud. Specific cases are described.

In some cases the piece represents the very end of a stolon, in others

it lay at a little distance from the tip. The differences in the behavior of these two kinds were found to be negligible.

(1) A piece cut off at a little distance from the tip and $1\frac{1}{8}$ mm. long when isolated, healed at each end within a few hours. It shrank slightly in both length and circumference within the first twenty-four hours, and formed a slight protrusion between the middle and one end within fifty hours. The piece was killed in Allen's modification of Bouin and cut into transverse sections of 5μ. The structure of this piece was found to be so similar to that of a normal budding stolon, at a corresponding stage, that special figures seem unnecessary. The facts are, however, stated and reference may be made to figs. 14, 15, 16. The ectoderm is completely healed over each end, but is columnar at only one end, the end nearer the bud. There is a septum but it is a little withdrawn from each end of the piece and, along its edges, from the dorsal and ventral ectoderm. Evidently the septum contracts as a result of the operation. At 50μ from the end the septum is a slightly thickened plate extending about half way across the haemocoele, with nuclei arranged alternately on the two sides of the plate. For the next 80μ the septum continues as a thin plate, showing in section a single row of nuclei. Following on this region the septum becomes thicker near one edge, showing in section a double row of nuclei, and forty-five micra farther on a slit-like cavity appears in this thicker portion separating two lamellae. The cavity grows in size as it is followed toward the other end of the piece, reaching a height of 92μ and a width of 24μ. Its entire length is 125μ. At no point does the cavity extend through the entire septum, i.e., a slender remnant of the original simple plate remains ventral to the vesicle as in normal budding. Throughout the extent of the vesicle lymphocytes and mesenchymal cells can be seen attaching themselves to its wall. Cells in the wall of the vesicle can be seen in mitotic division. The most obvious free blood cells of the haemocoele are of the highly specialized varieties, e.g., green cells, morula cells, and granular amoebocytes. These cells throughout the piece of stolon are found clumped and have an abnormal appearance as if beginning to disintegrate. Possibly they may be furnishing food for the growing bud. Mixed in with the abnormal cells can be seen the common type of tunic cell (compartmental amoeboid) but these usually appear normal. Practically all of the lymphocytes and mesenchymal cells have disappeared from the haemocoele; presumably they have been added to the septum to aid in blastogenesis. The few lymphocytes and mesenchymal cells which remain free in the haemocoele have the normal appearance.

Thus in this piece the septum aided by lymphocytes and mesenchymal cells, as in normal budding but in greater degree, has begun to form an endoblastic vesicle. Mitotic figures show that the wall of the vesicle is undergoing intrinsic growth. A noticeable difference from normal budding concerns the specialized haemocoelic cells. The beginning disintegration of these cells is correlated with the fact that the piece of stolon is cut off from the blood stream of the parent. Certainly the isolated piece of stolon encounters the problem of nutrition, and it would seem that this is solved by sacrificing the more specialized haemocoelic cells. At any rate the observations point in this direction.

(2) In another instance the abscised piece, 1.5 mm. in length, was taken well back of the stolonic tip. In four hours after isolation, healing of the ends had taken place and they were withdrawn slightly within the tunic. In twenty-four hours a slight swelling appeared on the side of the piece about one-half millimeter from one end. The piece was immediately killed in Bouin without acetic acid and serial sections of 5μ made. The ectoderm cells at each end are found to be cuboidal. Covering each end is a thin layer of new tunic. Within the haemocoele, at the end nearer the swelling, there is no sign of a septum for 30μ when it appears as a plate thickened near one edge and contracted away from the stolonic wall. The thickened portion shows at this level only a few nuclei and the cytoplasm no cell boundaries. In the thinner portion of the septum at this point there are no nuclei. Plainly the septum in this region has undergone contraction. This contraction of the septum away from the ectoderm is found to have occurred throughout the piece. It is greater at the ends than along the dorsal and ventral edges, and is especially great at the end farthest from the above mentioned swelling. For 145μ from this end the septum exists as a thin plate which occasionally shows a slight break and several irregularly thickened spots evidently formed by the addition of lymphocytes or mesenchymal cells from the haemocoele. The septum now becomes irregularly thickened near one edge (fig. 26). In this region there are some indications of amitotic cell division. Twenty micra farther on the enlarged portion of the septum acquires a fairly regular contour, triangular in the section and a good sized rounded cavity (fig. 27a). In the same region there can be seen in the haemocoele, beside the septum, a plate of cells similar to the septal cells in appearance and staining reaction. These are evidently mesenchymal cells or lymphocytes which have not yet added themselves to the blastogenetic mass (fig. 27b). The vesicular condition of the dorsal part of the septum continues for 225μ, beyond which

point the septum again becomes a single plate of attenuated cells with an occasional lymphocyte clinging to its sides. This condition persists through a stretch of 420μ and the septum then passes into a large terminal endoblastic vesicle which comes to an end 200μ from the actual end of the stolon (fig. 28).

This stolon-piece seems to be forming two endoblastic vesicles, one lateral, the other terminal; only the laterally placed vesicle could be recognized externally. One or the other or indeed both of these vesicles may have been present in an incipient stage when the piece of stolon was isolated, but their close proximity makes it very unlikely that both were forming at that time. Of course the terminal vesicle of the stolon-piece, if it did exist in the uninjured stolon, could only have existed as a lateral vesicle. The fact that the lateral vesicle is part of an externally perceptible bud may be taken to indicate that it is the older of the two. And the proximity of the terminal vesicle to this certainly suggests that the terminal vesicle is a new formation and not a mere modification of a lateral vesicle in existence before the operation.

(3) In a case very similar to the one just described, the abscised stolon-piece was only slightly over one mm. in length. This piece elongated very slightly and a lateral bud made its appearance. Sections reveal the presence of a very large lateral endoblastic vesicle in the recognizable budding area, and a smaller sub-terminal vesicle near one end of the piece. Within the cavity of the larger vesicle there are some free blood cells thus indicating that the vesicle may have been open to the haemocoele at some time. Gaps in the wall of the endoblastic vesicle sometimes occur in the natural budding process, and also free blood cells in the cavity of the vesicle. Evidently, then, free blood cells may enter the cavity from the haemocoele. Such gaps are evidently closed later on, either by intrinsic growth or by mesenchymal cells or lymphocytes. The free blood cells in the cavity of the vesicle are presumably absorbed. It may be noted in passing that the distinction between mesenchymal cells and lymphocytes is a formal one. Moreover, the similarity of these elements to the constituents of the septum as well as their fusion with the septum in regions of blastogenesis indicates that they all constitute a simple undifferentiated formative material the elements of which may assume different shapes and connections according to their temporary location, a point of view which is quite in accord with that of Brien and Brien-Gavage (2, 4). No definite organogenesis has begun in either of the two bud primordia of this piece, although the larger of the two is of a greater size than in the normal buds

at the time when organogenesis usually begins. The large vesicle does have its right side thickened preparatory to the formation of the endostyle. There are perhaps several reasons for the belated organogenesis of the larger vesicle, chief among which may be the lack of formative material which has perhaps been used up in the formation of the second and younger vesicle.

(4) Another stolon-piece, which was 1.3 mm. long when isolated, elongated slightly and budded in apparently normal fashion in about fifty hours. As the bud became noticeable the stolon contracted slightly in length and circumference. Examination of serial sections shows the following points. The ectoderm is stretched thin over each end of the haemocoele, and there is a delicate new tunic layer also covering each end. There is a well-formed, almost spherical endoblastic vesicle slightly nearer one end of the piece. No organogenesis has begun, but the entire wall of the vesicle is thickened as if in preparation for the process of rotation of the right wall to a ventral position (p. 199). Mitoses are numerous in the vesicle wall, and there are still irregular patches of lymphocytes and mesenchymal cells clinging to the wall where evidently they have recently migrated but have not yet been drawn into the wall itself (fig. 29, showing a part of the wall). The bud lies nearer one end, as said. In this part of the stolon the septum is still present in its usual plate-like form, but in the longer part of the stolon on the other side of the bud no septum is present. Here then it has evidently been entirely drawn into the endoblastic vesicle. Some of the more specialized blood cells of the haemocoele can be found clinging to the wall of the endoblastic vesicle in various places, but it is highly improbable that they are ever incorporated in the wall itself since no cytoplasmic connections are to be seen. The specialized cell most commonly attached to the vesicle wall is the familiar tunic cell, i.e., compartmental amoeboid. It is conceivable that they are concerned in the nutrition of the growing vesicle. At any rate such cells are apparently concerned in some measure with the general nutritive phenomena going on in the piece, for they are sometimes found free in the haemocoele with bits of other cells or entire cells in their cytoplasm (fig. 12a, b). This is evidently phagocytosis. Near one end of the haemocoele the compartmental amoeboid cells have formed a clump in which may be distinguished a few green cells apparently in the process of degeneration. Other small groups of blood cells show signs of degeneration, but there are many blood cells present that are perfectly normal in appearance.

(5) A stolon-piece 1.7 mm. long was cut out four or five millimeters

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back of the stolonic tip. Within the first two hours after isolation the piece shrank within the tunic and apparently healed at each end. Within the next twenty-four hours it elongated slightly. Within forty-eight hours a bud-like projection appeared on one side of the piece between the middle and one end. As the bud grew the stolon shrank. At the end of fifty-seven hours the piece was preserved in Allen's modification of Bouin and serial sections made. The ectoderm, as is usual, is columnar at each end. At the level of the bud-like projection there is a large endoblastic vesicle with a smooth regular wall thicker dorsally than elsewhere, and evidently close to the period of organogenesis. To it ventrally is attached a plate-like remnant of the septum. All this as in normal budding. The vesicle is prolonged toward one end of the piece into a septum, thin and plate-like ventrally, dorsally irregularly thickened and excavated by a cavity continuous with that of the main vesicle (fig. 30). This thickened part of the septum bears irregular projections and to it are attached strings of cells all evidently representing fusions of mesenchymal elements. Still nearer the end the septum ends much as in the normal, i.e., as a vertical plate thickest at its tip. On the other side of the bud there is no septum in the remnant of the stolon. Here then it must have been entirely absorbed in the endoblastic vesicle. In this part of the stolon too there are very few mesenchymal elements, apparently nearly all having been withdrawn into the blastogenetic center. The differences between the development of this piece and normal budding may be summed up: (1) The septum is absent on one side of the endoblastic vesicle, having been apparently drawn bodily into the latter. (2) The share taken by mesenchymal elements in the formation of the endoblastic vesicle is here (fig. 30) conspicuous. *It is clear then that the endoblastic vesicle is here developing less by growth than in the normal, and more by the direct utilization of material already present. And this statement applies to all the cases studied.*

(6) A piece 1.9 mm. long was cut from the distal end of an unattached stolon which exhibited no buds for a distance of 6–8 mm. Within the first twelve hours after isolation the piece grew one-half millimeter in length but decreased in width proportionally. The cut end, which apparently had healed completely, projected about one-fourth millimeter beyond the old tunic and was covered by a delicate new tunic layer. Within twenty-four hours after isolation a swelling appeared between the middle and the cut end of the piece, which was then killed in Allen's modification of Bouin and sectioned at 5μ.

In this piece there is a large endoblastic vesicle, close to organogenesis,

if indeed organogenesis has not already begun. And this vesicle, as in the preceding case, is prolonged on one side into a plate-like septum (about 470μ long) which, going toward the tip of the stolon, passes into a loose mass of rounded cells interconnected by cytoplasmic strands. This loose mass is quite long (240μ). Evidently there is here a deviation from the normal, apparently produced again by an increase in the passage of mesenchymal cells into the septum. Even where the septum is plate-like and thin, as occurs generally in these pieces, mesenchymal cells, are found attached here and there to it or fused in the shape of small plates close to it. In the wall of the vesicle are found not only mitoses but figures which suggest the occurrence of amitosis (fig. 31). Some free blood cells occur in the vesicular cavity as recorded for other cases. At the other end of the vesicle it also passes into a plate-like septum which extends toward the cut end of the piece. But this part of the septum is exceedingly short, only about 10μ long. A point may be mentioned in respect to the haemocoelic cells. The most abundant is the typical tunic cell or compartment cell some of which in this piece, both in the haemocoele and in the tunic of the tip of the piece, show a peculiarity not observed in any of the numerous preparations made either from normal stolons or from other cut pieces, although prepared with the same technique (Allen's Bouin, haemalum, acid fuchsin). The cells in question show red drops or vacuoles, which are larger than and do not resemble the granules brought out with neutral red in the living cells. George (6), to be sure, finds that the stainable granules in the living cell may agglutinate to form larger bodies.

Very little, if any, organogenesis has definitely begun in this young bud. In one spot near the anterior end of the vesicle a small group of lymphocytes has aggregated. This may possibly represent the earliest primordium of the dorsal tube. The primordium of the endostyle is also, it may be, represented on the thickened right side of the vesicle. The piece still has plenty of mesenchymal elements in its haemocoele.

In these longer isolated pieces of stolon, then, the bud primordium arises in the same general way as in the normal stolon. The septum shrinks from the sides (ends also, here) of the stolon, its cells multiply by mitotic and possibly amitotic division, and there are added to this septal mass lymphocytes and mesenchymal cells of the haemocoele. The differences are as follows. While in the formation of the endoblastic vesicle of the normal bud intrinsic growth of the septum and addition to it of mesenchymal cells both play a part, the symmetry of the growing structure (figs. 14, 15, 16) indicates that intrinsic growth is the dominant

factor. This results in the formation of a more or less symmetrical thickening which is then excavated. In the case of the isolated pieces, as has been said (p. 207) it would seem that the vesicle develops less by intrinsic growth and more by the direct incorporation of the septal material at the sides of the young vesicle and of mesenchymal elements of the haemocoele. Moreover, the section pictures, such as figs. 26, 28, suggest that the vesicle is sometimes formed, not by the excavation of a solid mass, but by the arrangement of mesenchyme and septum around a space. If this really occurs it is not surprising to find so often cells within the endoblastic cavity (fig. 31). Explanation of why two bud primordia form occasionally in such short pieces is not easy. In the normal, freshly collected material buds usually occur on the stolon at intervals of 1.5–2 mm. Some of the pieces which were only slightly over 1 mm. in length presented two buds primordia as has been described. The stolons from which these pieces were cut were usually unattached and without buds for a distance of 6–8 mm. Consequently there may have been present in these long barren areas an extra amount of reserve formative material, i.e., lymphocytes and mesenchymal cells, which may have been stimulated to action by the cutting, or by their freedom from the dominance of more highly organized centers, i.e., ascidiozooids. The matter of the nutrition of the young bud primordia is also problematical, but the discussion of this question is deferred.

Organogenesis in the longer pieces of stolon

In their further development the longer isolated pieces of stolon vary. In some of them organogenesis seems to proceed quite as in the normal bud, but in others the process differs considerably from the normal. Material for this portion of the investigation is not as plentiful as that for the study of the bud primordium, and there are consequently some gaps in the account. Specific cases are described.

(1) A stolon-piece, originally 1.5 mm. long, on the second day after isolation exhibited a normal looking bud borne laterally on the somewhat reduced stolon. Transverse sections of this piece reveal a well developed young bud in the stage of formation of dorsal tube and pericardial primordia. The dorsal tube primordium is a loose aggregate of a few lymphocytes 50μ long and lying in contact with the dorsal wall of the endoblastic vesicle near the anterior end of the latter. There is no organization as yet of this irregular mass into a rod or a tube. The primordium is very like the corresponding stage in a normally formed bud. The pericardial primordium adheres to the upper half of the right

side of the vesicle. It is a mass of loosely connected lymphocytes and the entire primordium has a length of 20μ. The vesicle itself has not undergone any torsion, although, from mid-region to the posterior end, the entire right side (future endostylar region), the dorsal wall, and the upper half of the left side are thickened, presumably in preparation for rotation. Throughout the length of the vesicle the entire septum has become two-layered, the cavity of the vesicle extending ventrally between the two thin septal layers. Lefevre (13) has recorded this condition and thinks the two layers eventually come together again. This case then reveals no recognizable deviations from the normal.

(2) A case in which the bud is slightly older than the preceding may be described. The stolon-piece, which was 1.7 mm. long when isolated, first shrank then increased slightly in length and appeared to bud normally. The piece was preserved about thirty-six hours after isolation. Sections show a well-developed bud. The primordium of the dorsal tube is 60μ in length, or about one-third the length of the entire vesicle, but as yet has not acquired a cavity nor has the ganglion been differentiated. This is about the condition of the nervous primordium in a normal bud at this stage. Left and right peribranchial chambers are forming although neither appears normal in size or method of formation. The right chamber is smaller than one would expect and exists as a tubular sac lying alongside the vesicle and anteriorly opening into the vesicle not far from the dorsal limit of the latter. Evidently there is some minor difference here from the normal method of formation. The left chamber differs from the right and from the normal. It exists as a short sac at the side of the vesicle, ending blindly in front, opening into the vesicle posteriorly. *Apparently in the formation of these chambers there are some real though minor growth differences from the normal.* It is surprising to find no sign of a pericardial primordium in this piece, and this point may be counted as a deviation from the type in the matter of times of formation of primordia. Whatever deviations from the type are exhibited in this piece are minor ones of morphogenesis. The cellular appearance shows that the bud is healthy.

(3) In another case the isolated stolon-piece was 1.7 mm. long. It failed to elongate, and curved roughly after the fashion of the letter U. After three days there was a swelling at each end. Sections show a well-developed terminal bud in the larger of the two ends, no septum in the intermediate region, and a loose collection of cellular plates and a few lymphocytes in the smaller end. In the bud of the larger end the pharynx is in communication with the two well-formed peribranchial

chambers by means of four series of stigmata. The oral and atrial siphons are both in process of formation, but no communication with the outside has been established. The dorsal tube primordium is now tubular and in communication with the pharynx. The ganglion has appeared as a small mass on the upper surface of the tube. The intestine is of considerable size and length and has begun to differentiate into regions; however, it has not yet established connection with the cloaca. The gut wall exhibits numerous mitoses. On the right side of the posterior part of the pharynx the pericardium and heart extend for a distance of 15μ. Some of the granular amoebocytes and green cells of the haemocoele appear in a degenerate condition; otherwise the cellular constituents look normal. *In this piece then there is terminal budding, and budding in the normal stolon of Perophora is lateral, more precisely dorsal.* In *Clavelina lepadiformis* budding is normally terminal: Brien and Brien-Gavage (3). Perhaps, however, the difference from the normal exhibited by this piece is not so radical since it may be that the cut was made at the exact spot of an incipient bud. The fact remains that the piece does exhibit terminal budding and that in correlation with this morphogenetic activity at one end the entire septum disappears, doubtless used up in the formation of the bud.

(4) Finally, there may be recorded a case at an advanced stage of development in which the organogenesis seems perfectly normal. When isolated the stolon-piece was 1.5 mm. long and $\frac{1}{2}$ mm. wide; it was not the end of a stolon. During the first twenty-four hours the cut ends healed completely and both extended beyond the old tunic covering; the length of the piece doubled in this length of time. At the end of the second twenty-four hours the length of the stolon-piece had doubled again and a bud was borne laterally about midway on the stolon. The bud was allowed to develop for another twenty-four hours before the piece was killed. Although relatively very large as compared to the stolon, the bud had not assumed visible activities. Since normally all buds become obliquely inclined to the stolon with apices pointing toward the end of the latter, the distal part of the stolon may be recognized even in an excised piece by the inclination of the bud. And so in this case the inclined bud served to show which was the proximal and which the distal end of the piece. The proximal end was unhealthy in appearance and practically devoid of free cells. The distal end had a few free cells in slow circulation. Sections show that the distal part of the stolon has a one-layered plate-like septum but its haemocoele is very poor in mesenchymal content. The part of the stolon on the other

side of the bud is 120μ long but the septum is absent from the terminal portion through a length of 50μ. The organs of the bud seem to be perfectly normal. Neither oral nor atrial siphon has broken through but in both cases the external invaginations of the ectoderm have met the pharyngeal and cloacal sacs. The dorsal hypophyseal tube opens into the anterior end of the pharynx and extends backward on the mid-dorsal surface of the latter for a distance of 50μ. A ganglionic mass lies dorsal to the tube for the greater part of its length. The peribranchial chambers are in communication with the pharynx by means of three tiers of branchial stigmata. A well developed heart is present for a distance of 45μ on the right ventral side of the pharynx. Its size and appearance are normal, and it probably was in action at the time the bud was killed as a slow circulation of cells was noted in the more healthy distal end of the stolon. Between stolon and bud the septum is just breaking its connection with the left peribranchial fold. In this particular case the haemocoele of both stolon and zooid is unusually poor in free lymphocytes and also in the specialized blood cells. *The remarkable feature exhibited by this piece is its great growth in length* and this may well be linked up with its paucity in blood cells at the time of fixation. They have been apparently largely used up.

Summing up it may be said that *these abscised pieces of stolon do exhibit in their development certain minor deviations from the type: a growth difference in the establishment of the peribranchial chambers; a difference in the relative times of formation of organ primordia; a difference in the location of the budding region, it being terminal in one case.*

Early behavior of the shorter pieces

Pieces of stolon 1 mm. or less in length, usually failed to elongate and began, at least, the process of direct transformation into an ascidian body. A few of these pieces increased somewhat in length, then shortened as the ascidian developed. Several stages in the process of development of these short pieces are individually described.

(1) A piece of stolon which was $\frac{3}{4}$ mm. in length when abscised healed at each end, shrank slightly in length, but increased in general diameter within twenty-four hours of isolation. One end of the piece appeared swollen within twenty-four hours and the piece was immediately preserved in Bouin without acetic. Sections show that ectoderm is complete at each end of the piece but columnar only at one end, the smaller. The septum has withdrawn for a distance of 100μ from this end, then appears in its typical plate-like condition, formed of a single layer of

cells. This condition persists, as the sections are followed forwards, for about 60μ when the septum is joined by a curved plate of mesenchymal cells, the two making a U-shaped structure as seen in section. This mass soon passes into an irregular structure (fig. 32) made up of a complex of trabeculae and the septum. That the complex is made up of plates attached to one another and the septum is obvious, and the histological structure shows that the plates represent fused mesenchymal cells. In many of the spaces between the plates are specialized blood cells, e.g., green and morula cells, which have been trapped during the formation of the complex. This irregular formative mass extends for a distance of 200μ toward the larger end of the stolon. In the 80μ between its termination and the end of the stolon there are only scattered cellular plates, a few free mesenchymal cells, and the specialized blood cells of the haemocoele. The majority of the specialized blood cells of the whole piece are packed closely around the irregular complex which is evidently to be looked on as an incipient blastogenetic mass.

Here then the entire septum has shrunk away from the ends toward the middle of the piece drawing with it practically all of the formative cells, thus giving rise to an irregular mass which doubtless would have developed further had opportunity been given. The green cells and compartmental amoeboid cells are the most numerous of the specialized cells. The latter have formed clumps at many points in the haemocoele. Many of these latter cells are exhibiting phagocytosis, the phagocytosed cell in most cases being one of the uncommon tunic cells (compare figs. 5 and 12a, b). Small groups of specialized cells in the haemocoele seem to be in the early stages of degeneration.

(2) Another short piece of stolon upon abscision was accidentally cut into two pieces, each less than one-half millimeter in length. These two pieces were allowed to remain very near together in the same tunic covering. After two days they fused completely and on the third day the fusion-piece had a smooth spheroidal contour and a very healthy green appearance (fig. 33). Sections show that the ectoderm is complete around the entire piece. Inside the haemocoele the majority of cells look normal and healthy. What doubtless is a remnant of the original plate-like septum is present (fig. 34). This lies in the central region of the mass. Near it and in part attached to it are some free mesenchymal cells, aggregations of mesenchymal cells, and little plates and masses obviously representing fused mesenchymal cells. There can be little doubt that this whole complex was in process of producing an endoblastic vesicle and not by the method of forming a solid mass which later is

excavated as is the case in normal budding (compare figs. 14, 15, 16). Some phagocytosis is going on. Of the phagocytes themselves some resemble compartmental amoeboids and others signet-ring cells (fig. 12a, b, c).

(3) Another stolon-piece, which was about 1 mm. long when cut, shrank about one-fourth millimeter in the first three hours after abscision, and at the end of twenty-four hours was only one-half millimeter in length and about as great in diameter. Sections show a complete covering of ectoderm, columnar at each end. In the haemocoele there is no definite septum. Instead there is found an aggregation of cell plates and cells surrounding in an irregular way a space or spaces (fig. 35) as if the formation of an endoblastic vesicle had begun in an atypical fashion (compare figs. 26, 28).

(4) Another piece which was only one-half millimeter in length when abscised was torn and bruised considerably in the cutting and was almost detunicated. Ten hours after isolation this piece had a curved or C-shape, was enlarged at one end, and had regenerated a new tunic. It was then fixed. Sections show that the ectoderm is complete around the entire piece. There is a recognizable remnant of the plate-like septum and this is connected with a large sac (fig. 36) the structure of which makes it almost certain that it has been formed not by the thickening of the septum followed by the excavation of the thickened part, but rather by an arrangement of cell plates and remnants of the septum around a space. To specify details, this conclusion is indicated by the very large size of the cavity and the thinness of the walls. Moreover there are enough blood cells in the cavity to be conspicuous. And, finally, the whole structure has been formed within ten hours. Many cases of phagocytosis are seen in the haemocoele of this piece. The phagocytic cells are of both the signet-ring and compartmental amoeboid types. The phagocytosed masses appear to be either bits of morula cells or of the more uncommon type of tunic cell.

(5) One short piece completed the formation of an endoblastic vesicle on the second day of isolation. When abscised this piece measured one-half millimeter in length and not quite one-fourth millimeter in diameter. Its length remained the same, but its diameter seemed to increase slightly, due perhaps to absorption of water. Sections show that the ectoderm is hardly complete over one end, but is entirely healed over the other. The end which failed to heal was bruised in cutting. There is a large endoblastic vesicle, 115μ long, 64μ high, 40μ wide. The entire wall is thin, the nuclei forming a single layer and showing mitotic figures.

There is only a short remnant of the septum, and there are very few mesenchymal cells in the haemocoele. This piece is, perhaps, less convincing than the preceding, but the large size of the cavity and the thin wall of fairly uniform thickness make it on the whole improbable that the vesicle has been formed in the typical way, i.e., by the establishment of a solid mass which is subsequently excavated (compare figs. 14, 15, 16). Many of the specialized cells of the haemocoele are in a degenerating condition; this is particularly true of the green cells, morula cells, and granular amoebocytes. The degenerating cells are closely packed around the greater part of the outside of the endoblastic vesicle and it is possible that some of this material may have been absorbed by the vesicle. Many signet-ring cells have solid inclusions in their vacuoles. Some compartmental amoeboid cells also exhibit phagocytosis.

Organogenesis in the shorter pieces

After the establishment of the endoblastic vesicle early organogenesis seems to proceed much as in normal budding.

(1) A stolon-piece three-fourths millimeter long when cut may be described. Within three hours after isolation this piece had healed at each end, had shrunk slightly within the tunic, and there was a decided difference in size in the two ends of the piece Within twenty-four hours after isolation a well-formed bud was borne very close to the distal end of the stolon, this end barely projecting beyond the bud, the diameter of the stolon was reduced to one-half its original size. Sections show a well-formed and very large endoblastic vesicle. A plate-like septum is present in the long part of the stolon and in the region of the bud a septal remnant extends ventrally from the endoblastic vesicle in the usual way. Close to the vesicle in their usual locations the primordia of the neural tube and pericardium are in process of formation. The dorsal tube primordium is as yet an unorganized mass, about 55μ long, irregular in shape in spots and again flattened, and not differing from the corresponding stage in the long pieces or in the normal bud. The pericardial primordium is about 40μ long and lies, as in the normal bud at this stage, high on the right side of the vesicle. This primordium is organized into the form of a flattened tube for about half of its length and thus is evidently developing as in the normal bud; as in the normal bud it is somewhat ahead of the dorsal tube primordium in organization. Both primordia are, in spots, closely applied to the wall of the endoblastic vesicle, but, as in normal budding, there is no definite

sign of cells passing from the wall of the vesicle into the primordia. In the haemocoelic cavities of both bud and stolon there are very few free mesenchymal cells or lymphocytes left, a fact which indicates that they were used in the establishment of the endoblastic vesicle and the organ primordia.

(2) In another case the stolon-piece, originally 1 mm. long, was allowed to develop four days before being killed. A bud of considerable size was formed; the stolon was reduced in length as the bud developed. Serial sections show a large pharynx from which the peribranchial sacs are being separated in the usual way, and there is an evagination, the esophagus, growing out from the left posterior region of the pharynx. The dorsal tube primordium is a compact, definitely outlined mass about 50μ long, in the usual position, offering no difference from the normal. In a normal bud of this stage of development the pericardial primordium is relatively large and has already become saccular. In the piece under study its only representative is a solid little mass of cells about 5μ long, lying however in the typical position of the primordium. The formation of the pericardial primordium is evidently greatly retarded in this piece. One may say that an attempt, so to speak, is being made on the part of the bud to develop its digestive and respiratory apparatus relatively faster than its circulatory system, whereas in normal development the primordium of the pericardium is one of the earliest to appear. A similar retardation in the development of the heart was described for one of the longer pieces of stolon.

(3) A final case may be described in which the piece of stolon was rather long, 1 mm., and in which the bud was allowed to develop into an ascidian body. Both ends healed within a few hours, and in the first twenty-four hours the piece showed a slight increase in length. A swelling appeared laterally near one end of the piece, and as the swelling increased the stolon shrank in length correspondingly. The bud was allowed to develop for thirty-six hours before being killed in Bouin. At this time the bud was 112μ high and 144μ wide; the total length of the stolon only 345μ; the bud lateral and nearer one end of the stolon. Sections show that the septum has disappeared throughout the stolon except at one end where there is a little remnant, 40μ long. Very few mesenchymal cells are left. Evidently then the bulk of the septum and mesenchyme went into the formation of an endoblastic vesicle and the other internal primordia. The following organs are present and in fairly normal states and relations; pharynx, gut, peripharyngeal chambers, cloaca, nervous system, pericardium and heart. The gut has not

yet reached the cloacal wall; however it is already differentiated into three well-defined regions. Atrial and oral siphons have not completed their formation, yet both are indicated. Branchial stigmata are not yet formed, although the inner wall of the peribranchial chamber is in contact with the pharyngeal wall in many places.

DISCUSSION AND CONCLUSIONS

1. In the non-blastogenetic parts of the stolon of *Perophora viridis* the septum is a thin plate representing a single layer of cells. In the establishment of an endoblastic vesicle the septum takes the leading part. In this blastogenetic process it thickens in its dorsal region, largely, it seems clear, as a result of intrinsic growth, and secondarily by the addition of some free mesenchymal elements. This symmetrical dorsal thickening then becomes hollowed out to form a vesicle which may or may not locally involve the entire septum (figs. 15, 16).

This description of the septum and formation of the endoblastic vesicle agrees essentially with that given by Brien and Brien-Gavage (4) for *Perophora listeri*. It differs from that given by Kowalevsky (10), Ritter (15), and Lefevre (13), who describe the septum as primitively double and the endoblastic vesicle as arising by a growth process which is virtually an evagination.

2. In the case of the longer isolated pieces of stolon (1.1 to 1.9 mm. in length) the external appearance would indicate that the internal morphogenetic processes were very similar to those of normal budding. There are, however, differences of some interest. Section pictures show that the dorsal region of the septum thickens, but less by growth than by addition of a considerable amount of mesenchyme both in the form of cell plates and little cell masses. In this way an irregular blastogenetic mass is produced (fig. 30) into which a large part of the extra-blastogenetic portion of the septum is drawn (p. 207); however there is usually a recognizable remnant of the septum left in the stolon on one side of the vesicle. In these pieces, then, it seems that the formative materials (septum and mesenchyme) are making an adaptation in harmony with a decreased food supply. The intrinsic growth of a localized region of the septum being checked, the nearby formative material is directly drawn upon to a greater degree than in the normal.

3. The differences between morphogenesis in the very short isolated pieces of stolon (1 mm. and less) and in the normal bud are striking. The typical process of formation of an endoblastic vesicle mainly by local growth of the septum is distinctly departed from. In such a piece

in fact the septum may be difficult to recognize, for it may either be broken into small remnants not distinguishable from similar mesenchymal plates (fig. 35) or greatly obscured by the addition of plates and masses of such cells (fig. 34). The microscopic pictures are quite opposed to the idea that these formative elements unite to form a thickened mass which is later hollowed out to form a vesicle. No such mass is found. On the contrary pictures indicate that the plates of formative tissue (septum and mesenchyme) combine at first in a very irregular way to enclose a space or spaces (figs. 34, 35). By a process of rearrangement these irregular plates may form, it is thought, large vesicles with fairly uniform walls (fig. 36), such as are sometimes produced within a very short time after isolation (10 hrs. in one case) of the piece. Some of the section pictures of longer pieces (fig. 26) suggest that in them also the septal and mesenchymal constituents may arrange themselves around a space present from the beginning.

This interpretation of the microscopic pictures seems to be the only justifiable one. It is clear that the morphogenetic process in these little pieces, not only cut off from the parental food supply, but left with a minimum of formative material, undergoes a striking and what may be regarded as a qualitative change. The endoblastic vesicle, it would seem, is established in the quickest and easiest way by assembling together all of the available formative material into an arrangement which, though rough, has from the start the idea of a vesicle.

There are some slight differences between the process of formation of an endoblastic-vesicle, as outlined here, and that described by Brien (2) in the regeneration of experimentally isolated stolonic tips of *Clavelina lepadiformis*. In these abscised stolon tips the formative elements are the same as in the Perophora stolon, namely, septum, mesenchymal cells, and lymphocytes. But the septum first contracts strongly (effect of the cutting), and to the contracted septum are added other formative elements, the whole making a solid formative mass which is subsequently hollowed out to form an endoblastic vesicle. Brien finds that in normal budding of Clavelina the septum breaks up into its constituent cells which later aggregate along with the other formative elements to form a solid blastogenetic mass which is then hollowed out to form the inner vesicle of the ascidiozooid. While the departures from the typical morphogeny exhibited by the isolated pieces of Perophora are much greater in the short than in the longer pieces, they are in them all of such a kind as to warrant looking on them as adaptive.

4. In the case of the longer pieces the development is one of mixed

growth and morphallaxis. In the case of very short pieces development is close to if not entirely pure morphallaxis. Wilson (21) records that occasionally such a little piece may entirely transform into an ascidian, the little remnants of the stolon persisting in such cases as have been here described being completely retracted into the body. In the parallel case of the transformation of pieces of Clavelina stolon Della Valle (5) found that a very small piece might be wholly transformed into an ascidian body, while in the case of a larger piece a part (terminal) becomes body and a part persists as stolon. Della Valle regards the morphogenetic processes in the latter case as closer to regenerative budding than to morphallaxis.

5. In the processes of organogenesis the majority of isolated pieces differ less from the normal buds than in the establishment of the endoblastic vesicle. The two most noticeable differences are concerned with the retardation of development of the heart primordium and with the smaller size and somewhat different method of formation of the peribranchial sacs. The former difference is probably correlated with the lack of sufficient formative material.

6. The formative material consists of septal cells, mesenchymal cells, and lymphocytes, and, as has been said (p 205) the distinction between these elements is a formal one. The significant fact is that they are all simple undifferentiated elements, very unlike the highly specialized and conspicuous green and morula cells which take no part in the formative processes.

Lefevre (13), while first to demonstrate conclusively that, in the ascidian bud, free cells of the blood aggregated to form organ primordia, nevertheless failed to identify these cells as any particular kind of blood cell.

Schaxel (16) states that it is the undifferentiated cells, to be found within the ectodermal and entodermal epithelia, and among the mesenchyme (blood cells) of the isolated branchial basket of *Clavelina lepadiformis* which are solely responsible for the regenerative process. Before the regeneration occurs, he believes that all specialized tissues disintegrate leaving only undifferentiated cells of the three layers. These regenerative cells, he finds, retain, as they proliferate, their original relative positions, i.e., ectoderm outside, entoderm inside and mesenchyme between the two. Thus a three-layered sac is produced which is, as he says (loc. cit. 139), quite like the stolon of a budding region.

Spek (18), on the contrary, thinks that the totipotent regenerative elements in the isolated stolon tips (winter buds) of *Clavelina lepadifor-*

mis are certain special amoeboid drop-cells. He also assigns a nutritive rôle to these cells under certain conditions. He finds that the drop-cells are manufactured in the wall of the mid-gut.

Brien and Brien-Gavage (2, 3, 4) find that the formative regenerative tissue in both *Perophora listeri* and *Clavelina lepadiformis* consists solely of the unspecialized cells, that is, the septum, mesenchyme, and lymphocytes, and, as said, they make no great distinction between these elements. The results of this investigation are in accord with those of Brien and Brien-Gavage in this matter. Brien (2) has found Spek's drop-cells in Clavelina and has analysed their inclusions as glycoproteins. He thinks that these cells may play an important trophic rôle in the budding or regenerating stolon but are not formative.

Wiegand (20), in his experiments on Clavelina, in which the upper part of the body is regenerated from the visceral sac, finds that in the regenerating region Spek's cells are absent, although abundant elsewhere. They cannot, therefore, be looked on as the regenerative elements. Wiegand, again, was unable to find in the visceral sac cells corresponding to Schaxel's indifferent reserve cells which the latter regards as the regenerative elements. As to the actual origin of the regenerative cells in this process, Wiegand came to no conclusion.

7. Cells exhibiting phagocytosis appear frequently in the abscised pieces, rarely in the normal stolon. Brien (2) finds in two of the Clavelinidae both in normal budding and in regeneration that certain large phagocytic cells are a constant and abundant element in the haemocoele of the stolon. He thinks these phagocytic cells are preying upon the more specialized cells of the stolon which have begun histolysis.

ABSTRACT

In normal budding of the social ascidian, *Perophora viridis*, the inner vesicle of the young bud is formed chiefly by local intrinsic growth of the stolonial septum. The septum itself is not a two-layered structure, that is, virtually a closed sac, but a simple partition representing a single layer of cells. To the growing septum, mesenchymal itself in origin, are added a few of the free unspecialized mesenchymal cells of the stolonic cavity, the two combining to form a symmetrical thickening in the dorsal region of the septum. This mass is excavated to form the cavity of the inner vesicle of the bud.

Isolated pieces of stolon when transforming into small ascidiozooids depart from the normal morphogenetic method employed in budding, the departure being greater in short (1 mm. or less) pieces than in

longer (1–2 mm.) pieces. In longer pieces the blastogenetic region of the septum may grow slightly but it depends largely on the incorporation of extra-blastogenetic parts of itself and on the addition of free unspecialized mesenchymal cells in attaining the vesicular state. In the short pieces the septum as such is scarcely recognizable. The section pictures indicate that remnants of it and mesenchymal plates and masses arrange themselves around a space to form the wall of a vesicle. The establishment of an endoblastic vesicle out of formative material already present instead of its establishment by a process of true growth, i.e., formation of new tissue, is a process of morphallaxis as contrasted with ordinary regeneration.

The formative elements in both budding and morphallaxis are the unspecialized undifferentiated cells of septum and blood stream.

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EXPLANATION OF PLATES 15–17

(All figures, unless otherwise stated, were drawn under oil immersion at a magnification of 1490 and with the aid of a camera. The original figures have been reduced by two-fifths.)

Abbreviations Used in Figures

ectd .....ectoderm
end. ves, end. v. .....endoblastic vesicle
fus. pc.....fusion piece
haem.....haemocoele
mes.....mesenchyme
mes. c.....mesenchymal cell
mes. pl.....mesenchymal plate
mes. proj.....mesenchymal projection
mes. m.....mesenchymal mass
n. t. pr.....neural tube primordium
pc. pr.....pericardial primordium
sep.....septum
sep. rem., sep. r. .....septal remnant
si.....sinus
tun.....tunic
tun. c.....tunic cell
vac.....vacuole

PLATE 15

Fig. 1. Photograph of a colony of *Perophora viridis* grown on a slide in the laboratory. About natural size.
Fig. 2. Cross section of a stolon in a non-budding region, 750 ×.
Fig. 3. Part of the end of a living stolon stained in weak neutral red showing the more common type of tunic cells with granules, and the thick ectoderm.
Fig. 4. Fixed and stained tunic cells of the more common type, (a) from the haemocoele, (b) and (c) from the tunic.
Fig. 5. Tunic cells of the more uncommon type, fixed and stained.
Fig. 6. Green cells, (a), living; (b) and (c), fixed.
Fig. 7. Morula Cells, (a), living; (b) and (c), fixed.
Fig. 8. Signet-ring cell, fixed.
Fig. 9. Granular amoebocyte, fixed.
Fig. 10. Lymphocytes in varying aspects, fixed.
Fig. 11. Mesenchymal cells, fixed.
Fig. 12. Phagocytes from regenerating stolon pieces, fixed.
Fig. 13. Transverse section of distal end of septum in a normal stolon.
Fig. 14. Transverse section of thickened or blastogenetic septum just proximal to the tip.
Fig. 15. The same septum from a transverse section of a still more proximal region of the stolon. A cavity has been hollowed out in the thickened dorsal portion.
Fig. 16. The same septum, from a still more proximal transverse section, at the place of fullest extent of the endoblastic vesicle of the bud. The vesicle does not involve the entire septum.

PLATE 16

Fig. 17. From a transverse section of stolon; (a) an endoblastic vesicle which has involved the entire septum; (b) a plate of mesenchymal or formative cells in the haemocoele beside the endoblastic vesicle.
Fig. 18. Septum as seen in transverse section, showing a single row of cells (nuclei), with mesenchymal plate projecting from the side.
Fig. 19. Transverse section of thickened or blastogenetic septum exhibiting mitotic division and showing mesenchymal cells being added to it.
Fig. 20. Semi-diagrammatic transverse section through a stolon and young bud showing the endoblastic vesicle and the earliest organ primordia: neural tube and pericardium (due to reversal under the microscope the left side of the figure, with the pericardial primordium, represents the right side of the bud) × 170.
Fig. 21. Dorsal portion of the picture shown in figure 20, enlarged to show the neural tube primordium.
Fig. 22. Portion of the left side of fig. 20 enlarged to show the pericardial primordium.
Fig. 23. From a transverse section, showing two blood sinuses in the ventral region of a young bud.
Fig. 24, a, b. The two divisions of the stalk which connects bud and stolon, as seen in transverse section.

Fig. 25. The undivided part of the stalk, close to the stolon, as seen in transverse section.

Fig. 26. From a transverse section of one of the longer abscised pieces of stolon, showing the septum in early blastogenetic activity. Note its irregularly thickened dorsal region and the mesenchymal cells being added to it.

Fig. 27. From a section of the same series as fig. 26: (a) endoblastic vesicle, an irregular asymmetric mass; (b) a plate of mesenchymal cells close beside the young vesicle in the haemocoele.

Fig. 28. From a section of same series showing large terminal endoblastic vesicle.

PLATE 17

Fig. 29. From a transverse section of one of the longer abscised pieces. Part of the wall of a large endoblastic vesicle is shown. Attached to its outer surface are two little masses of mesenchyme and a remnant of the septum.

Fig. 30. From a transverse section of one of the longer pieces of stolon, showing an early stage in the formation of an endoblastic vesicle. To the irregular, excavated mass representing the vesicle are attached a considerable remnant of the septum and an equally large mesenchymal plate, *mes. proj.*

Fig. 31. Endoblastic vesicle, inclosing some haemocoelic cells, as seen in transverse section of one of the longer pieces of stolon. Mitosis is in progress in the cells of the vesicle wall.

Fig. 32. Very irregularly formed blastogenetic mass. From a transverse section of a short piece of stolon.

Fig. 33. Surface view of a regenerative mass, *fus. pc.*, resulting from the fusion of two very short abscised pieces of stolon, still in the old tunic, *tun.* × 50.

Fig. 34. From a transverse section through the fusion-piece shown in fig. 33. Septal remnants, mesenchymal plates and masses fusing around a cavity.

Fig. 35. From a transverse section of a short abscised piece of stolon, showing irregular formative mass made by the fusion of septal remnants and mesenchymal cells.

Fig. 36. Large irregularly shaped endoblastic vesicle, as seen in transverse section of one of the shorter pieces of stolon.

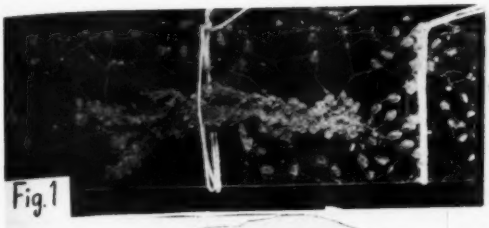


Fig. 1

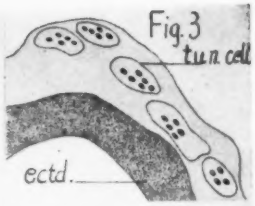


Fig. 3

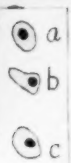


Fig. 5

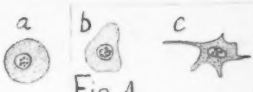


Fig. 4

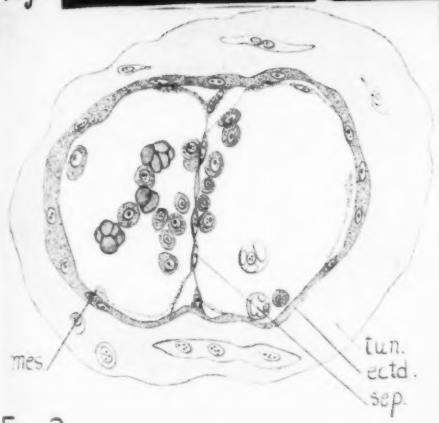


Fig. 2

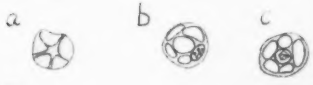


Fig 6

Fig. 7

Fig. 8

Fig. 9

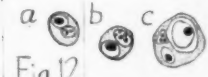


Fig. 12

Fig. 10

Fig. 14

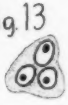
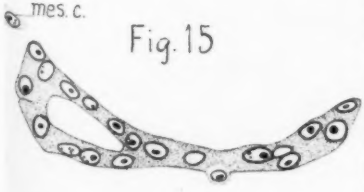


Fig. 13

Fig. 11



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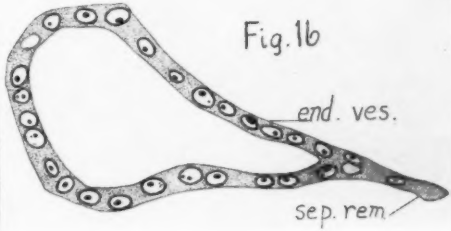

Fig. 16

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Fig. 17

Fig. 18

Fig. 19

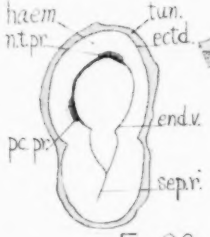
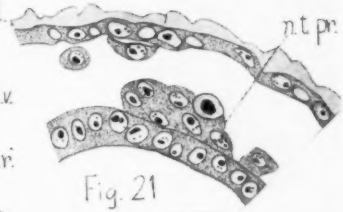


Fig. 20



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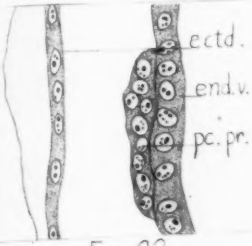

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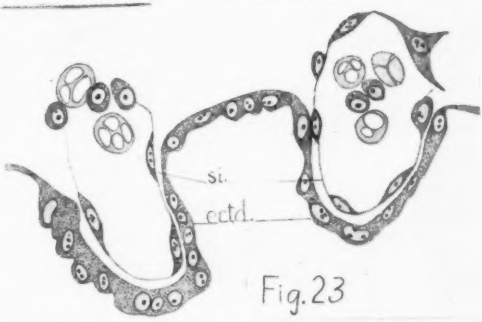

Fig. 23

Fig. 24

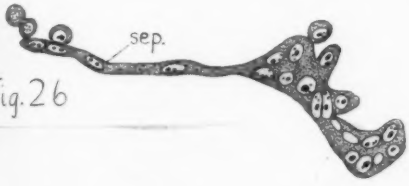

Fig. 26

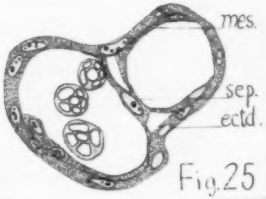

Fig. 25

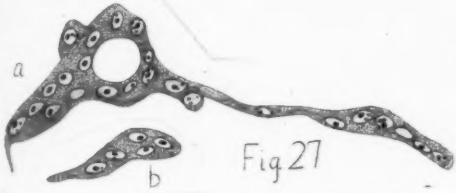

Fig. 27

Fig. 28

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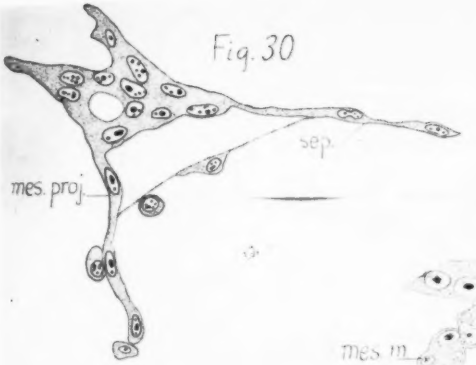
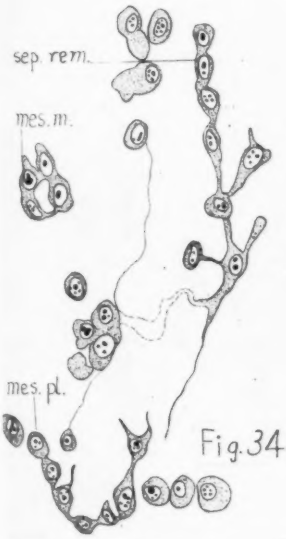


Fig. 30

Fig. 33

Fig. 29



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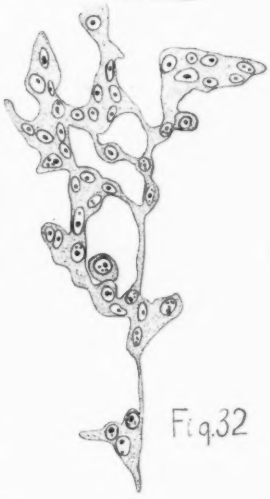


Fig. 32

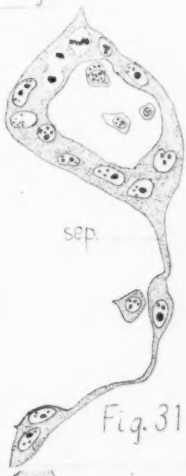
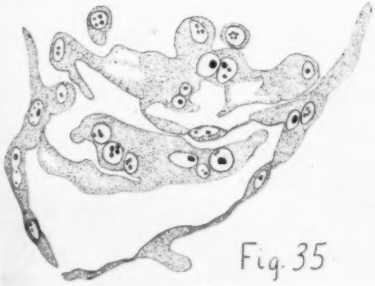


Fig. 31



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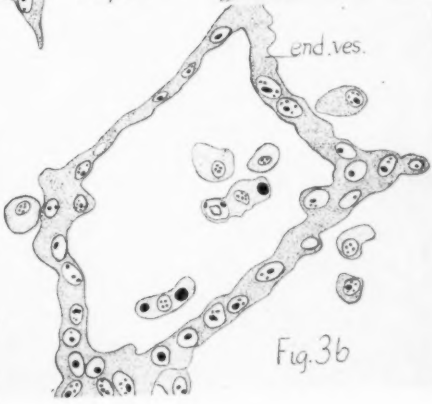

Fig. 36

THE OLD FIELD PRISERE: AN ECOLOGICAL STUDY

By W. M. Crafton and B. W. Wells

Plates 18–20 and 1 Text-figure

INTRODUCTION

In the eastern mesic forest region the old field vegetation is an ecological unit strikingly distinct from that of the climatic climax vegetation. The primary stages of the developmental vegetation, the prisere, consist of an herbaceous flora entirely. This is followed by the tree flora which constitutes the dominant natural vegetation of the eastern United States.

The herbaceous flora, composed of grasses and weeds, ranges throughout old fields and waste places in a more or less regular series. In the southern upland bare areas the most frequent pioneer is the crab grass (*Syntherisma sanguinale*). It is particularly abundant in recently cultivated fields. It enters during cultivation and maintains dominance rarely after the second year. The general seeding and growth of invaders result in the exclusion of this annual. These invaders—tall weeds (*Eupatorium*, *Aster*, *Solidago*, and others)—play the dominant rôle for a time. A few years later the old field vegetation changes again and the third phase is initiated. This time broom-sedge (*Andropogon* spp.) invades—reaches its extreme expression—then passes out with the ecesis of pines (*Pinus taeda*, *P. echinata*, and *P. virginiana*).

Where the tree vegetation is open in stand in the old pine fields, broom-sedge is well represented. On the other hand, where the pines form dense consocies by mass invasion this grass soon becomes only a relict of the final prisere stage.

The rate of establishment of the elements of the prisere are profoundly influenced by the edaphic and aerial conditions. Further, the sequence, in part at least, bears a direct relation to the ecesis of the final prisere element. This study deals primarily with the habitat in relation to the distribution of the vegetation. With this approach, certain conclusions can be drawn to account for the definite stages in the prisere.

It is not intended to discuss or even mention all of the plants in the old fields. The species under observation proved of interest because of the

aspects they present, importance in the habitat analyses, and, of course, their direct relation to the prisere.

METHOD

The studies of this problem were confined to the fields about Raleigh, North Carolina. For the measure of plant invasion and competition meter quadrats were marked off in several communities of different soil habitat. The vegetation in the quadrats was recorded late in 1930 and early spring, summer, and fall of 1931. Bare areas were made in the spring of 1931 by spading up meter square plots. Records of plant growth were obtained as in the other quadrat areas. In many of the quadrats, 20 cm. intra-quadrats were marked off. This permitted closer checking of the effect of competition. Further, a rather detailed study of a terraced slope was made.

The water supplying power of the soil was obtained only for comparative purposes. In certain striking situations texture analyses of the soil were made. Further, relative light values were obtained. All of these data were gathered from the various representative communities.

Information in regard to seedling growth and response to drought proved significant in the interpretation of the stages of the prisere development. This information was obtained from studies in both the green house and the field.

Close observation of the old field vegetation coupled with the data gathered from the above sources comprised the mode of attack on this problem.

THE VEGETATION

Like all primary vegetation, that of the old fields is somewhat xeric in nature. The upland areas tend to display a great many species adapted to the xeric habitats; the lowlands contain those species adapted to the more mesic conditions.

Usually the vegetation in either situation is so variegated that the casual observer sees nothing but a heterogenous mass of grasses and weeds. However, close and frequent observations will disclose a rather graded series of species in the revegetation of old fields. The appearance of this series is related to the nature of the soil and the species involved.

Successional stages of the old field associes

The pioneer consocies. It is a common observation that cultivated fields become covered with crab grass. Bermuda grass (*Cynodon*

Dactylon) is less common as a dominant. Here and there occasionally appears *Paspalum* sp., but its occurrence is rare. In local moist areas cockle bur (*Xanthium commune*) and smart weed (*Polygonum* spp.) often become subdominants or even dominants. However, in upland fields they are always minor elements. In eroded fields where the subsoil is exposed, the pioneer stage is represented by ragweed (*Ambrosia artemisiifolia*), button weed (*Diodia teres*), and poverty grass (*Aristida* sp.). These exposed clay areas and the *Cynodon* consocies have the effect of holding up the usual rapid development of the prisere.

The annual crab grass often retains its dominance for a second growing season. At this time horse weed (*Leptilon canadense*) is found in addition to the minor plants of the previous year (pl. 18, fig. 1). This is the

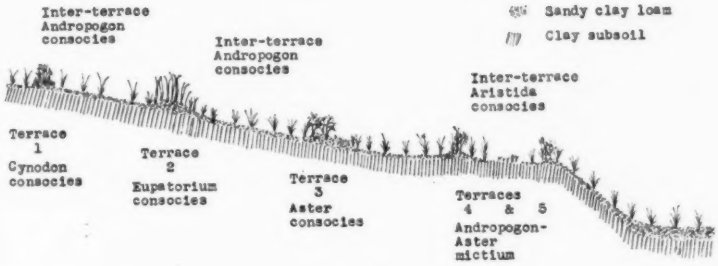

FIG. 1

most conspicuous plant of the associes because of its great height. Aster or some other tall weed may become well represented at this time, or at least the year following. Orchards, cultivated three or four times during the growing season, display crab grass and scattered aster. Foxtail (*Chaetochloa* spp.) is often present. Fields from which small grain crops have been harvested in the early summer, are given over to these grasses. Other fields which have reached the weed stage prior to planting to small grain sometimes skip the crab grass stage. Such fields are usually disked. After harvest the weeds become dominant again. Crab grass and foxtail may be found scattered throughout these areas representing remnants of the true pioneer stage. In sections of the upper piedmont it is a fairly common practice to prepare the fields in the summer for fall sowing. The plowing is usually done in July. With a few rains the fields become covered with crab grass.

Other plants in recently cultivated areas are mostly annuals. Occa-

sionally a few perennial weeds become established, but they are never abundant. Even the regular minor elements are more or less widely scattered.

The most striking aspects in this stage are contributed by toad-flax (*Linaria canadensis*) and tall sheep sorrel (*Rumex stricta*). In the sandy regions of the state they present beautiful vernal aspects of lavender and crimson. Fields cultivated the previous year are often covered by either or both of these plants. However, in the piedmont they are less common. Ox-eye daisy (*Chrysanthemum Leucanthemum*) and ragwort (*Senecio Smallii*) appear in the pioneer stage only in Bermuda grass areas which have been established for several years (fig. 2). Further, these species are much more common in the piedmont and mountain areas.

LIST OF THE CHARACTERISTIC PLANTS OF THE PIONEER CONSOCIES

Dominants

Ambrosia artemisiifolia
Aristida sp.
Cynodon Dactylon
Paspalum sp.
Syntherisma sanguinale

Subdominants

Chaetochloa glauca
Chaetochloa viridis
Leptilon canadense

Minor plants

Allium sp.
Amaranthus retroflexus
Amaranthus spinosus
Antennaria plantaginifolia
Bidens spp.
Brassica sp.
Chenopodium album
Chenopodium Botrys
Chrysanthemum Leucanthemum
Convolvulus arvenis
Coreopsis sp.
Datura sp.
Diodia teres
Erigeron spp.
Eragrostis sp.
Hypericum gentianoides
Linaria canadensis
Oenothera laciniata
Oxalis stricta
Panicum capillare
Panicum virgatum
Rubus sp.
Scleranthus annuus
Sida spinosa
Solanum carolinense
Xanthium commune

The tall weed consocies. This is the intermediate stage, composed mainly of aster (*Aster* spp.), golden rod (*Solidago* spp.), dog fennel (*Eupatorium* spp.), sneeze weed (*Helenium tenuifolium*), and Carolina golden rod (*Euthamia caroliniana*). The last three are often dominant in neglected pastures. With grazing these weeds retain prominence

for years. However, when this factor is withdrawn, broom-sedge assumes dominance. This grass, though long seeded, is apparently held in check by grazing.

Frequently one observes succession within a weed consocies. In the coarser soils dog fennel may be followed by *Aster*. However, the former may not be entirely eliminated until the final stage is initiated.

In the open weed communities the dominants of the pioneer stage strongly resist the invaders. These grasses are best represented in short weed consocies, that is, communities of Carolina golden rod and sneeze weed. On the other hand, where the plant cover is heavy there is only occasional evidence of the retiring species.

A longer time is required for the suppression of Bermuda grass than any other of the pioneers. In many cases it is represented throughout this stage as an underplant community. It is entirely eliminated only in dense stands of weeds. Otherwise, it may persist long after broom-sedge has taken possession of contiguous areas.

Of all these weeds, the dog fennels (*E. hyssopifolium* and *E. capillifolium*) are probably the most important ones which extend far into the coastal plain. *Solidago odora* appears in the lower sand regions where clay is near the surface. *Aster ericoides*, most common in the piedmont, also disappears in the sandy regions of the state.

In open weed communities, daisy fleabane (*Erigeron* spp.), rabbit foot clover (*Trifolium arvense*), and hop clover (*T. agrarium*) represent the spring aspects. However, where the weed stand is dense these plants are apt to be eliminated.

LIST OF THE CHARACTERISTIC PLANTS OF THE TALL WEED CONSOCIES

Dominants

Aster ericoides
Aster Tradescanti
Eupatorium capillifolium
Solidago odora
Solidago rugosa

Subdominants

Achillea millefolium
Eupatorium hyssopifolium
Eupatorium sp.
Euthamia caroliniana
Helenium tenuifolium

Minor plants

Antennaria plantaginifolia
Bidens spp.
Daucus carota
Desmodium acuminata
Desmodium sp.
Erigeron spp.
Lactuca canadensis
Lespedeza striata
Lespedeza procumbens
Trifolium agrarium
Trifolium arvense
Rubus spp.
Senecio Smallii
Solanum carolinense

The broom-sedge consocies. This final consocies of the prisere may be found in various stages of expression. In different fields it is often observed sharing an area with tall weeds, short weeds, pines, or almost by itself (figs. 3, 4, and 5). With tall weeds it represents the part of an invader. Where the short weeds are found a more advanced stage is represented. The extreme expression of dominance is reached when even the short weeds are excluded. This latter situation, however, is of rare occurrence.

A few differences in the distribution of the tall weeds in the piedmont and coastal plain have been pointed out. The broom-sedge stage in these two sections of the state is represented mainly by *Andropogon virginicus*, *A. Elliottii*, and *A. argyraeus*. However, in the coastal plain *A. glaucopsis* becomes prominent in addition to *A. virginicus*.

In connection with this stage it is well to mention the contributors to the spring aspects. Fleabane (*Erigeron* spp.) and ragwort (*Senecio Smallii*) appear locally in broom-sedge areas. The appearance of rabbit foot clover and hop clover is more or less limited.

LIST OF CHARACTERISTIC PLANTS OF THE BROOM-SEDGE CONSOCIES

Dominants

Andropogon Elliottii
Andropogon virginicus

Subdominants

Andropogon glaucopsis
Eupatorium sp.
Euthamia caroliniana
Solidago sp.

Minor plants

Andropogon argyraeus
Antennaria plantaginifolia
Aster grandiflorus
Chamaecrista nictitans
Chrysopsis graminifolia
Daucus carota
Desmodium acuminatum
Desmodium sp.
Erigeron spp.
Eragrostis sp.
Gnaphalium purpurea
Helianthus sp.
Hypericum gentianoides
Lactuca canadensis
Lespedeza augustifolia
Lespedeza procumbens
Physalis sp.
Senecio Smallii
Sieglingia seslerioides
Smilax rotundifolia
Smilax Bona-nox

Seedling growth

Seedling growth is of great importance in regard to the rôle of plants in any sere. While the juvenile growth alone is not an adequate index of ecesis, knowledge of this phase in vegetational studies is paramount.

In the early period a slight shift to the dry soil condition may easily prove fatal. With rapidly growing species, however, the importance of such a change is less significant. Likewise, plants growing in shade may be affected to a less extent at such times.

Experiments. Seedlings of aster, golden rod, broom-sedge, and crab grass were placed in competition in several pots of loam soil and subjected to artificial drought. The tall weed and broom-sedge seedlings were 1 month old. The crab grass was less than 2 weeks old. After 1 week with no water the weeds could not be revived. Only 2 of the 6 broom-sedge seedlings survived sufficiently to start growing again. One of 2 crab grass seedlings lived. At the end of the week without water, the leaves of crab grass were only rolled; those of the other plants were rolled and dried. Seedlings in other pots subjected to the same drought treatment, but removed from the direct sunlight, rolled after 2 weeks.

Two pots of 14 day old broom-sedge seedlings were placed in the shade of potted plants in the greenhouse. Two other pots were not shaded. The shaded plants lived 3 days without water. The seedlings left exposed to the direct sunlight died the first day.

Bermuda grass and broom-sedge seedlings were grown in the same pot. The plants were not watered through the day as is necessary for the perpetuation of broom-sedge in the open. The Bermuda grass showed no indication of a drought condition, but the broom-sedge died.

The apparently slow growth of the weeds and broom-sedge is of importance. After 6 weeks of growth the leaves of broom-sedge averaged 2 cm. in length. Tillering was very pronounced at this time. Weeds of the same age were twice as tall. Crab grass in the period of 6 weeks flowered at the height of 25 cm.

Field observations. Seedling crab grass and weeds are rarely found before the middle of May. Broom-sedge, on the other hand, may be found in abundance by the first of the month. The oldest seedlings at this time may be about 3 weeks of age. By the middle of May there are very few small seedlings surviving in the bare areas. The rains become more and more infrequent and the critical periods—a few days without rain—come on and only the oldest seedlings are able to endure.

Broom-sedge seedlings literally cover the ground in lowland areas. The frequency with which they occur here is much greater than in the adjoining upland regions. In the latter situation the leaves may roll after a few days with no rain. However, under the same conditions those seedlings which grow near clumps of weeds or under the leaves

of broom-sedge do not appear to be affected. Search for seedlings of this plant in Bermuda grass consocies revealed not a trace of their presence.

THE HABITAT IN RELATION TO THE VEGETATION

The bare sunny areas give rise to those species which are usually photophilous. However, from the standpoint of xerism the exposed subsoil and sandy clay loam soils are occupied by very different pioneers, and changes in the vegetation progress at different rates. Where the surface soil is eroded, leaving the red subsoil exposed, changes in soil conditions must be brought about before the broom-sedge can successfully ecize; or more correctly, the habitat must be made favorable for the successful invasion of the tall weeds. Usually in these areas the nutrient-water complex operates. The only change produced in the habitat of the loam and sandy soils is that induced by the tall weeds. The soil water conditions are much more favorable at this time for seedling establishment. This can be shown to be of vital importance to broom-sedge. However, the influence of texture becomes a factor even in this regard.

Variations in soil and aerial habitats are often clearly shown on slopes. And the vegetation of the old fields shows marked response to the water, texture, and nutrient conditions often present in such areas.

The prisere stages are always altered by the depth of the surface soil. In deep sandy soils the advance is rarely as rapid as in those more shallow.

Quadrat studies

Bare area quadrats. Meter square areas were spaded up in various field communities in the early spring of 1931. The vegetation in these quadrats was recorded twice (early summer and fall) during the year.

On a sandy clay loam upland area, Bermuda grass became the dominant element, while ragweed played the rôle of the subdominant. A plot was located in an area dominated by the grass just mentioned. In June 3 ragweed seedlings were growing well in the 20 cm. intraquadrat. By fall only 1 attenuated plant remained, while 8 grass plants had appeared.

In another Bermuda grass consocies, bared plots gave rise to a luxuriant growth of crab grass. Smart weed and cockle bur were minor elements. This area was of sandy clay, but in lowground. Crab grass became the dominant element in an upland sandy loam area. Yellow foxtail played the minor part.

The bared plot in an *Andropogon* consocies gave rise to 43 ragweed and 24 Carolina golden rod plants by June. In the fall 34 ragweeds remained. These ranged from 15–60 cm. in height. At the same time the golden rod numbered 23. Some showed definite suppression. Not all of these flowered. The height of these plants ranged from 10–40 cm. Crab grass was represented by 53 hardy plants. *Lespedeza procumbens*, though reduced in numbers, was still present in the fall. In the 20 cm. intra-quadrat ragweed and *Lespedeza* paid the heaviest toll to competition. Of 6 *Lespedeza* seedlings in June, 2 survived until fall; and of 4 ragweed only 1 survived. All 6 crab grass plants flowered.

The open plot in poverty grass was in contrast to the above mentioned area. This quadrat was located higher on the same gentle west-facing slope. Other than the few inches of sandy clay, the soil is of heavy red clay. The soil habitat is in direct contrast to the lower part of the slope which is of deep sandy clay loam.

By late May, 13 plants grew in the quadrat. One week later there were 114 seedlings. Though *Lespedeza* and ragweed had increased numerically, bracted plantain (*Plantago aristida*) was dominant. In contrast to this there were 400 seedlings in the lower quadrat. By fall *Lespedeza* was reduced from 29 to 6 plants and ragweed from 19 to 10. The mortality of all seedlings was greater in the stony sandy clay soil as compared to those in the loam soil. Crab grass was represented by only a few plants.

Crab grass became the dominant element in the bared areas in *Eupatorium* and *Helenium* consocies. Bermuda grass and sneeze weed were the minor elements. The soil in these contiguous consocies was of the sandy clay loam type.

It is commonly observed that where the Cecil clay subsoil appears at the surface the vegetation is scant. Poverty grass and button weed usually dominate such areas. And Carolina golden rod is often scattered over the clay surfaces. For experimental purposes soil from a *Cynodon* consocies was removed to an "outcrop" of the red clay. Where fertilizer was applied with the transported soil, ragweed and crab grass grew. There were but 3 plants of the crab grass. These plants did not appear elsewhere on the galled area.

Aristida consocies. This particular area had been dominated by poverty grass for a number of years. The soil to the 6 inch depth is of sandy clay. Beneath this depth a heavy red clay sub-soil is found. The entire area is so stony that it was almost impossible to obtain soil point data.

The spring vegetation consisted of the usual hop clover and bracted plantain. This was followed by rabbit foot clover.

In the fall of 1930 four asters flowered. The following spring 16 rosettes appeared, but these were reduced to 8 by the middle of June. At this time the average height of these plants was 10 cm. Only 4 of the 8 plants flowered the next fall. It may be well to call attention to the fact that in deep sandy loam soils aster had reached the height of 25 cm. by the first of May. This contrast is no more striking than that of the rabbit foot clover in various localities. It should be stated that this clover grew to the average height of 20 cm. in this consocies. Where manure had been applied the plants averaged 45 cm. in height.

Cynodon consocies. This upland area of sandy clay loam had been uncultivated for 4 years. In the quadrat 3 asters flowered in the fall of 1930. Early the next spring 34 rosettes of this plant were growing. By June this number had increased to 38. However, only 7 of this number survived and flowered in the fall. The height of these plants ranged from 25–40 cm. This species of aster (*A. ericoides*) normally grows much higher.

Aster consocies. This area apparently had been in Bermuda grass a few years previously. It indicates directly what will happen in the *Cynodon* consocies in the next few years. This *Aster* consocies was on a north-facing slope of shallow sandy loam.

The vegetation in the fall of 1930 was represented predominantly by aster (*A. ericoides*) and Bermuda grass. By May of the following year rabbit foot and hop clover covered the area. A month later the vegetation was changing to aster; Canada lettuce, ragwort, and wild carrot were the minor elements. Bermuda grass was reduced from 23 to 4 plants in the course of a year. During the same period aster was eliminated to almost half of the 1930 growth. Of 57 plants in June, only 14 flowered in the fall. On the other hand, broom-sedge increased from 6 plants to 8 in the course of the year. The tussocks did not develop to great size.

Solidago consocies. The soil condition is the same as that in the *Aster* consocies. The quadrat was approximately in the same location.

By the middle of May the plants in this consocies had reached the average height of 25 cm. A month later it was definitely affecting field sorrel (*Rumex acetosella*) and daisy fleabane (*Erigeron ramosa*). Of the 69 weeds present in June, 51 flowered in the fall. Sensitive pea (*Chamaecrista nictitans*), Bermuda grass, and beggar-ticks (*Desmodium acuminata*) were much suppressed. Only the *Desmodium* flowered.

Syntherisma-Helenium associes. These plants were the main elements in a pasture. The soil is a sandy clay loam.

In spite of the evenly scattered sneeze weed, crab grass was still dominant in the fall of 1931. The minor element was represented by Bermuda grass. Both dog fennel and broom-sedge were invading this area. However, the former was most aggressive.

Eupatorium consocies. This community occupied the deep sandy loam area near the *Syntherisma-Helenium* associes.

There was no change in this area over the one year period. However, it was of interest to note the prevalence of *Lespedeza,* but the absence of crab grass. Sneeze weed was sparingly represented. Broom-sedge was much better represented in this community than in the previously mentioned associes.

Solidago-Andropogon mictium. The soil type of this area is a deep sandy loam.

A record of the 1930 growth was not obtained from this area. Of the 38 golden rod plants which appeared in June of 1931, only 21 remained until fall. The broom-sedge maintained its representation of 16 tussocks.

Euthamia-Andropogon associes. The sandy clay loam soil of this area is 60 cm. deep.

Other than the changing spring vegetation of hop clover and rabbit foot clover this area remained the same throughout the growing season. Many of the golden rod plants reached only 20 cm. in height. On the other hand, broom-sedge grew to the maximum height of 70 cm.

Andropogon consocies. This community was on an upland area of red Cecil clay.

Of the 25 tussocks in the quadrat only 10 flowered in 1930. About half of this number flowered the following year. By June of 1931 several aster plants appeared. These were entirely eliminated during the summer.

Summary of quadrat studies

The greatest number of a single species that appeared in the bared areas was crab grass. It grew abundantly on lowland and upland loam soils. On the clay soils this grass was meagerly represented. These areas of thin soils gave rise to ragweed and poverty grass.

The persistence of crab grass was observed to be most pronounced in the short weed communities. In areas dominated by tall weeds it was rarely found. Conversely, broom-sedge was best represented in the

tall weed communities. The study of the *Cynodon* consocies indicates the slow ecesis of *Aster*. A further stage of this was demonstrated in the *Aster* consocies in which Bermuda grass was definitely suppressed. The increased representation of broom-sedge in the same quadrat points to the trend of the succession. The establishment of broom-sedge with resultant suppression of weeds was indicated in the *Andropogon-Solidago* quadrat. The competition of tall weeds with broom-sedge seemed to be more intense than that in weed consocies.

From the standpoint of soil conditions, it will be sufficient to point out here, that the succession rate seems to be retarded most on the exposed subsoil. The pioneers on these areas are of the xeric type. On the rather shallow or deep loam soils the usual pioneers are present.

Terraced slope transect

This particular study will bring out three major influences which retard the prisere. However, the same factors which operate to produce this effect are general and important for all the situations as they exist in the ordinary development of the prisere.

Except for a few areas of exposed subsoil, the field is sandy clay loam from 2 feet to 6 inches in depth. The greatest depth of the surface layer is on the terraces and at the foot of the slope. Below this range is the red subsoil.

The inter-terraces were rather uniformly covered by broom-sedge; the only exceptions being the galled areas on which bracted plantain and poverty grass grew (text fig. 1). Boneset (*Eupatorium* sp.), golden rod (*Solidago* sp.), and cudweed (*Antennaria plantaginifolia*), which usually accompany broom-sedge, were well represented throughout the field. Other minors were beggar-ticks, sensitive pea, tickle grass, Carolina golden rod, aster, golden rod, and dog fennel. At the base of the slope the mesic *Andropogon glaucopsis* was co-dominant with *A. virginicus*. But on the slope this more mesic species thinned out to only occasional tussocks of reduced size. Trailing bramble and other species of *Rubus* were well represented in the low ground. These ranged half-way up the slope and were most prominent on the terraces.

The series of terraces presented a peculiar distribution of old field vegetation. The first of the 5 terraces considered was occupied by Bermuda grass as dominant, with aster (*A. Tradescanti*) subdominant. The upper part of the second terrace was crowned by dog fennel (*E. capillifolium*) (fig. 6). In a small area golden rod (*Solidago odora*) was dominant. On the lower end of this same terrace, which gradually dis-

appeared, was aster. Broom-sedge was dominant where the terrace completely faded out. On the third terrace aster played the dominant rôle in a local area, but broom-sedge was invading this consocies. Again, broom-sedge was dominant where this terrace leveled to the slope. Broom-sedge, aster, and Bermuda grass occupied the two remaining ridges with bramble, golden rod, and Carolina golden rod as minors.

Thus, where the terraces were still prominent, Bermuda grass and weed associes registered dominance. However, where the ridges faded out broom-sedge—tall weed mictia were found. The ecotone was abrupt on the upper side of each terrace; on the lower side weeds and broom-sedge mingled (fig. 6).

In the spring the terraces were densely covered with rabbit foot clover. This plant was practically confined to the ridges. On the lower side of the "aster terrace" (No. 3) vetch was the spring dominant. The ecotone between this species and broom-sedge was very definite. By fall this area was given over to Bermuda grass with aster only a minor element (fig. 7).

Several changes occurred the following year ('32). Bermuda grass no longer was dominant. It was replaced by aster. The dog fennel on the second terrace gave way to aster also (figs. 8 and 9).

Water relations

The importance of water in plant ecesis cannot be overemphasized. The expression of this factor is closely related to texture. And texture plays a very important part in nutrient relations as well.

Water supplying power of the soil. For the measurement of this factor Livingston soil points were used. The points were inserted at the 15 cm. depth and removed at the end of an hour. The following data were obtained from the several communities on the slope. The readings are given in milligrams.

	May 29 (5 days after rain)
Eupatorium consocies	600
Andropogon consocies	710
Cynodon consocies	980
Andropogon-Aster associes	930
Aristida consocies	540

Texture. The rôle this factor plays in water relations is of major importance. This is particularly true for plants in the juvenile stage. Samples for analyses were taken from the surface and the 15 cm. depth.

TABLE 1

	DOG FENNEL	ASTER	BROOM-SEDGE		ASTER	BERMUDA GRASS	ANDROPOGON-ASTER ASSOCIES	BASE OF SLOPE
Coarse	18.10	13.27*	14.15†	10.00	12.80		9.76	
Medium	14.00	5.41	2.94	5.45	4.00		7.33	
Fine	12.12	3.50	3.80	7.05	6.42		8.10	
Very fine	42.17	48.00	53.70	55.00	52.00		40.15	
Clay	13.40	29.56	24.00	22.20	24.00		34.21	
	99.79	99.74	98.59	99.70	99.22		99.55	
Coarse	18.30	13.35	12.90	7.00	7.95	9.30	6.60	7.00
Medium	0.00	4.00	3.30	0.00	3.30	3.00	2.15	6.80
Fine	3.50	7.20	4.39	6.00	4.60	4.00	9.40	12.50
Very fine	49.00	53.50	51.80	49.60	55.13	55.00	52.10	46.00
Clay	28.60	21.85	27.10	37.15	28.80	28.50	29.34	27.45
	99.40	99.90	99.49	99.75	99.78	99.80	99.59	99.75

* Near terminus of terrace.
† At terminus of same terrace.

TABLE 2

	ANDROPOGON CONSOCIES	EUPATORIUM CONSOCIES
Coarse	13.27	13.40
Medium	6.20	7.25
Fine	8.54	9.75
Very fine	42.31	42.53
Clay	29.45	26.65
	99.77	99.58
Coarse	7.85	9.25
Medium	5.82	8.73
Fine	8.43	11.70
Very fine	38.65	40.64
Clay	38.40	29.22
	99.15	99.54

The tables give in percentages the various grades found. The first set of figures is the surface soil analyses (table 1).

The texture data (table 2) further emphasize the relation of texture

to the ecesis of broom-sedge. In the *Andropogon* consocies, dog fennel was still subdominant. Joining this area was the *Eupatorium* consocies with a few broom-sedge tussocks. These communities were not on the terraced slope. Soil for analyses was taken from the surface and 15 cm. depths. The first set of figures (table 1) is the analyses of the surface soil.

Light

A stop-watch photometer was used to determine this factor. The method of procedure and calculation recommended by Clements[1] was used. A series of exposures was made on a clear day at noon. This gave the standard, or the most intense light values. Exposures were then made in the various communities. All data except that from the *Vicia* socies were obtained in September at one-half the height of the vegetation. The exposure in pine was made by directing the aperture of the photometer so that the light which filtered through the branches would strike the photographic paper.

The relative average values are given below.

	per cent
Dog fennel	9.5
Aster	12.2
Bermuda grass	8.5
Vetch	1.7
Pine	2.5

Summary of habitat studies

In the coarser soils capillary activity is comparatively less than in the fine soils. Wells and Shunk[2] demonstrated this to be the case in their studies of the sand hill region of the lower coastal plain. That the water supply is tied up with texture is fairly well demonstrated by the results in the Raleigh region.

The *Eupatorium* consocies gave the lowest water supplying power on the basis of texture. The soil texture is of a coarser grade than that of the other communities. In the *Cynodon* consocies and the *Andropogon-Aster* associes the soil point readings approached equal values. The texture is approximately the same for these two communities. The

[1] Clements, Frederic E. *Plant Physiology and Ecology*. Henry Holt and Company, New York, 1907.

[2] Wells, B. W., and Shunk, I. V. The Vegetation and Habitat Factors of the Coarser Sands of the North Carolina Coastal Plain. Ecological Monographs 1: 465–570. Oct., 1931.

Aristida consocies gave the lowest water supply value. In this case the soil type was compact red clay. The water either penetrates to only a slight extent or it is held more firmly by the soil particles than in other soils. The low reading in broom-sedge may be accounted for by the fact that the soil points were on the border line between the surface sandy clay loam and the red clay layers. In addition to this, all of the communities except the *Aristida* and *Andropogon* consocies were heavily covered by spring plants. In the contiguous *Andropogon* and *Eupatorium* consocies (not on the slope) we notice only slight differences in the texture. But the finest textured soil is in the broom-sedge area.

The significant facts brought out by these data are: the relation of texture to water as indicated in the two *Eupatorium* consocies, and the low water supplying power of the raw red clay soils in the *Aristida*.

The greatest response to the light factor was found in the dense stand of *Aster* on the third terrace. Bermuda grass seemed to be unable to grow to any extent in this situation. However, it is of interest to note that the dense shade of the early vetch had no effect upon the later development of this grass. In open consocies of Bermuda grass growth begins early in the spring. On the other hand, in the vetch community by the latter part of May there were but few plants arising from the underground stems. Apparently after the death of this legume, Bermuda grass grew rapidly.

The aster, dog fennel, and Bermuda grass communities gave approximately the same light values. Nevertheless, the shade produced by the former seemed sufficient to reduce the grass considerably.

The light value under pine is relatively low as it filters through the branches. This effect is minimized by the reflected light which enters under the branches. Broom-sedge shows little response to this factor where the tree vegetation is not crowded.

The density of broom-sedge tussocks is dependent upon the water supply. Since they are a foot or more apart, light could never become a factor. Those plants which ecize in an *Andropogon* consocies require a maximum amount of light, but their water requirement is probably low. Cudweed, beggar-ticks, sensitive pea, etc., are to be found in the otherwise bare spaces between the broom-sedge tussocks. Especially to be mentioned are the pines which constitute the next stage in the sere.

Ecads

The response of plants to several known factors can often be readily determined by field observations. With the soil water and the light factors playing a decisive part in the establishment and maintenance of

the principal old field elements, it seems desirable to present data concerning the certain responses to factor changes.

Broom-sedge. The distribution of the leaves on the flowering stalks were not as abundant nor as large in the *Eupatorium-Andropogon* complex as in the *Andropogon* consocies. However, the coloration was good. The flowering stalks were of the average height but very slender. Further, the number of stalks produced were comparatively few. All of these conditions indicate insufficient water and low light. Water was probably the most influential. The texture studies in this area are helpful in the interpretation of the water-texture relationship. This is especially important in relation to seedlings.

Broom-sedge under pines showed severe suppression. In such instances, it was probably due to root competition since the plants showed no evident elongation response to the lessened light.

Dog fennel. The ecads of this plant showed definite suppression on the inter-terrace where broom-sedge was dominant. Not only were the stems reduced in size, but those which grew from the rosettes were fewer in number. In many cases the number of stems was decreased and the size reduced over the number at the same rosette of 1930. Where three stalks of the 1930 growth could be counted only one grew in 1931. Suppression is most noticeable where the dog fennel grows very near the broom-sedge tussocks. In areas where broom-sedge has become well represented, a few minutes' search will disclose many such situations.

Aster. The effect of competition for water was very noticeable where this representative grew with broom-sedge. The leaves were not so numerous nor large as in the associes. Occasionally several stems grew from a single rosette. This happened only in the more open spaces between the broom-sedge tussocks. Suppression was greatest where the two species grew closely together. Golden rod made a similar response in competition with broom-sedge.

Bermuda grass. By contrasting the plants in the consocies with those in the *Aster-Cynodon* mictium the effects of competition become very noticeable. Line transects of 40 cm. lengths in the consocies gave an average count of 8 plants. In contrast to this, transects in open aster gave an average count of only 4 plants. In the dense stand of aster an occasional spear of grass could be seen. These plants had fewer leaves, and the stems were unusually long.

DISCUSSION

Inhabitants of an area become established because of adaptations to the conditions present in that particular habitat. The habitats of bare

and covered areas are, of course, very different so far as the water and light conditions are concerned. Evaporation of water from the soil surface on a bare area is much greater than that where vegetation has developed. And the height and density of the vegetation become factors in the maintenance of surface soil moisture. The evaporating power of the air in such instances is decreased due to the check of wind velocity and solar radiation.

The above facts form a basis for the concepts to account for the vegetational sequence in the prisere. A crab grass community is best represented on bare areas. In competition with open communities of annuals it will maintain dominance. We notice that crab grass holds on for considerable time in the short weed community of *Helenium*. And when opened to light, the small-grain fields often become vegetated with this grass. However, in areas where perennials are dominant, though the light is favorable, this grass is not found. Low water during drought periods is probably the limiting factor, because dominant plants are always as fully represented as the habitat and the time permit.

The rapid growth of crab grass is responsible for its ready appearance in cultivated fields. The hydrophilic condition of colloidal particles in the large water-holding cells may contribute a great deal to its maintenance in open ground. The weeds grow more slowly and are more susceptible to drought in the seedling stage. Further, cultivation may hinder the growth of these plants should they appear. Just why tall weeds do not always become dominant the season following abandonment is not certainly known. The general seeding is probably such that they should become dominant at this time. Sometimes this is the case. In other situations the number of tall weeds that ecize the first season are few. This has been observed on both thin stony soil and lowland loam. A second season after abandonment a weed community is usually found. The number of the previous year is increased both by seeding and by the appearance of plants from the stolons of those already established. The resulting shade eliminates the crab grass.

Bermuda grass loses out to tall weeds, because of reduced light, also. This was clearly seen in the transect study. In the *Aster* consocies, which shaded into the *Cynodon* consocies, there was a decrease in the representation of the grass. A further stage was shown on the fourth terrace in which broom-sedge and aster mingled and Bermuda grass was a minor underplant. Invasion into Bermuda grass by weeds requires a much longer time than entrance into one of crab grass. This is related to the growth habits of the grass species. The annual crab grass has no chance with a perennial species. Bermuda grass, a peren-

nial, competes successfully with weeds until considerable shade is produced. It is then suppressed.

The invasion of broom-sedge into old fields rests upon the establishment of more mesic conditions induced by the tall weed flora. Pot experiments have shown broom-sedge seedlings to have a slow growth rate and to be very sensitive to a slightly dry soil condition. In the field it has been observed to germinate and grow in the open, but in a few days without rain the seedlings disappeared. However, shaded seedlings withstood the adverse conditions better. The concept advanced here is that the soil in closed tall weed communities does not dry out so rapidly between the spring rains so that broom-sedge can become established. The various situations on the terraced slope intensify this concept. In the finer soils where aster had suppressed the Bermuda grass, broom-sedge was ecizing. On the coarser soils (terrace 2) the broom-sedge was scarcely represented. It can never enter the *Eupatorium* consocies until there is almost daily rainfall through the spring. The coarse, surface soils dry out much too rapidly for the slow-growing grass to become established. This characteristic is probably responsible for the minor representation of *Andropogon virginicus* in the "sand hills." Similar response to texture difference was noted in another *Eupatorium* consocies and the joining *Andropogon* consocies. The water relations as influenced by texture could have been the only factor operating against the uniform establishment of the broom-sedge.

The ecotone between broom-sedge and Bermuda grass is definite where these two grasses inhabit contiguous areas. The former is rarely found with Bermuda grass. The ecotone between a field of crab grass and one of broom-sedge is just as sharp. If the invasion of broom-sedge was due solely to its apparently slow growth in the juvenile stage, the tall weeds and this grass would appear simultaneously. This does not occur. Not until the weeds have become well established does broom-sedge invade. And we notice that it rarely enters communities of annual weeds. This can be explained by the comparative growth of the annual and perennial weeds in relation to their influence on the soil and aerial habitats during the spring season. Annuals do not grow to any extent until the middle of May. Summer annuals probably do not germinate before that time. Thus, the shade produced by these plants is not sufficient to keep the surface soil from drying out before the intermittent drought periods of early summer come on. On the other hand, the tall weeds which arise from perennial roots and reach an average height of 25 cm. by the first of May, create a different moisture condition in the surface soils. The surface soil in plant communities of this

height does not dry out appreciably in the short time between rains. The rainfall from April 26 to June 15 in 1931 averaged 1 rain every 3 days. The growth of broom-sedge under the weed cover is usually possible throughout May at least. The increasing infrequency of rains after this time would tend to reduce seedling establishment. The shade of the weeds introduces another factor which may be advantageous to the broom-sedge. Transpiration from the seedling grass may be reduced considerably. The low light value in shaded communities is apparently of little consequence to the broom-sedge.

Once established under these conditions, the grass is able to compete with the weeds. The root system so thoroughly ramifies the soil that the weeds are finally eliminated. The keen competition broom-sedge offers was indicated in the reduction of the weeds in the aster quadrat. Wherever broom-sedge is found with other herbaceous vegetation the severity of competition may be seen. And it is only under the influence of the tree vegetation that broom-sedge, once established, shows marked suppression.

The exclusion of the many minor species from the tall weed communities is probably due to their intolerance to low light. Many of the same plants that are found in open fields, appear again in broom-sedge communities. Their representation is always of minor importance. Cud weed, boneset, sensitive pea, and Carolina golden rod are never found in closed communities. While not all of these appear in open fields they often are present in broom-sedge fields. That Bermuda grass is a strong competitor for the fullest expression in an area, is shown by the absence of these plants. The habit of growth is probably the limiting factor. Many of them are annuals. The early growth of Bermuda grass might be sufficient to eliminate these plants. Of the perennials, the Carolina golden rod is known to begin growth comparatively late in the spring. Its possibilities of survival are best in broom-sedge, which seems to develop less rapidly than the Bermuda grass. The growth habit and the water factor probably account for the appearance of these plants in the one open community and their absence in the other.

Another influence in the distribution of vegetation, often present in fields, is the galled areas. The nutrient-containing soil is eroded in such instances, and the soil which remains is such that very little water penetrates. Consequently a major part of the water that falls on these red clay surfaces quickly runs off. Hence, the plants growing on the galled areas must be very xeric in nature.

Where poverty grass or button weed is found in abundance it is safe

to conclude that the soil habitat is of an unusually dry, sterile type. These plants play the dominant rôle year after year. They are freed almost entirely from competition with broom-sedge, and only gradually are weeds able to ecize. Even then, the number of these invaders does not increase to any extent over a period of years. When low nutrient and low water factors are involved jointly, the change in vegetation is brought about by improvement of the texture and the nutrient conditions. This, of course, requires a considerable lapse of time.

It should be mentioned that where the subsoil appears on the level, revegetation is more rapid. Nutrient accumulates more rapidly, and more favorable water conditions exist in these situations than on slopes.

SUMMARY

1. There are three definite stages in the revegetation of abandoned fields in the vicinity of Raleigh, North Carolina. While there are a few variations in the species' representation throughout the state, the general trend is the same. The pioneer stage may involve several species. On the thin soil, poverty grass, ragweed, and button weed become the dominants. The more fertile soils become vegetated commonly with crab grass. Bermuda grass is less common. The intermediate stage is always represented by the tall weeds. The several species occupy most any soil habitat. It seems that dog fennel is most particular and favors the sandy soils. Broom-sedge becomes established following the ecesis of weeds. Usually the pines follow this grass.

2. Studies of seedling growth showed very clearly why the three stages in the prisere exist.

3. Quadrats established in various plant communities gave an index to the nature of competition and the resultant successional trend in the prisere. A terraced slope transect introduced a number of differentials which were unusually helpful in carrying out the analysis of priseral development. The texture-water complex proved most important here.

4. Soil point data showed no great difference in the water supplying power of the soil in the several communities. The very coarse soil gave a low reading. The soil point data from red clay soils show clearly why the more xeric plants become the pioneers.

5. The light values were relative. There was a considerable response to reduced light in the *Aster-Cynodon* mictium. And where this factor was not involved in annual short weed communities, crab grass held on.

6. Ecads proved valuable as indicators of small variations in the habitats.

7. This study leads to several definite conclusions: The various soil habitats produce no important changes in the priseral sequence. The rapidity with which crab grass grows, and its ability to withstand longer drought periods than the other species, is responsible for its position as the pioneer. The slower-growing weeds can not become established before the year following abandonment. Slow seedling growth and susceptibility to prolonged drought are probably most influential. The modification in the surface soil moisture brought about by the tall weeds makes possible the invasion of broom-sedge. While the series may be retarded for a time, as soon as the tall weeds ecize abundantly enough to decrease the water loss from the surface soil, broom-sedge will become established. Broom-sedge does not enter bare areas, nor areas with short vegetation, because of the sensitiveness of the seedlings to even slightly dry soil conditions. Both crab grass and Bermuda grass are affected by reduced light. Hence, the establishment of weeds means the elimination of these pioneers. The suppression of the weeds is due to the competition set up by the perennial root system of the broom-sedge.

EXPLANATION OF PLATES

PLATE 18

Fig. 1. Syntherisma-Leptilon associes.

Fig. 2. Chrysanthemum socies in a Cynodon consocies.

Fig. 3. An Andropogon consocies. Boneset and red-stemmed golden-rod frequently hold on to the end of the prisere.

PLATE 19

Fig. 4. Ecizing pines in broom-sedge. The flowering plants at the extreme right are asters. The prisere advance has been held up because of the persistence of Bermuda grass.

Fig. 5. Mature pine forest with broom-sedge thinning out under the influence of the trees.

Fig. 6. The sole occupant of the upper part of this terrace was dog fennel. Notice the reduced size of the plants growing with broom-sedge.

PLATE 20

Fig. 7. Cynodon consocies on the lower side of the third terrace. Aster became dominant the following year.

Fig. 8. Aster consocies where Bermuda grass was dominant the preceding year.

Fig. 9. Aster consocies on terrace 2, which has replaced a dog fennel (*Eupatorium capillifolium*) consocies in one year.

PLATE 18

1934
MANHATTAN
KANSAS

1

2

3

LIBRARY
MAY 14 1934
MANHATTAN

PLATE 19

4

5

6

LIBRARY
MAY 14 1934
MANHATTAN
STATE COLLEGE OF AGRICULTURE AND APPLIED SCIENCE
KANSAS

PLATE 20

7

8

9

THE SIPHONAPTERA (FLEAS) OF NORTH CAROLINA, WITH SPECIAL REFERENCE TO SEX RATIOS

By Archie D. Shaftesbury[1]

Two Text-figures

Several years ago, I noticed a marked preponderance of females among the fleas (Order Siphonaptera) collected each year by the zoölogy students at Woman's College, Greensboro. However, I assumed that this was likely due to selection of the larger specimens, which were the females, or to capture of the slower ones which might possibly be the gravid females, and so paid no particular attention to the differential sex ratio among these insects until April 1930, when I examined carefully a freshly killed gray squirrel (*Sciurus carolinensis carolinensis*) and found 31 specimens of the squirrel flea, *Orchopeas wickhami*, which included 23 females and only 8 males. Since I had combed the squirrel very carefully, and had, so far as possible, secured all the fleas, large and small, the question was suggested as to whether this apparent differential sex ratio might not be a real one. The result has been the collection and examination, during the past three years, of a great number of fleas, including over five thousand specimens from common animals in various parts of North Carolina.

[1] I am indebted to many biology students at Woman's College, Greensboro, for aid in the collection and preparation of specimens, particularly to Misses Ida Katherine Allen, Maude Ashworth, Annie Laurie Bason, Mary Brummitt, Virginia Cohoon, Catharine Cox, Constance Herritage, Dorothy McGhee, Ferne Mitchell, Iris Nelson, Jessie Parker, Kathleen Parker, and Catherine Taylor, and also to Elmer E. Brown and Frank Brown who have sent to me a considerable number of fleas from North Carolina and other states. I wish to thank Mr. C. S. Brimley, assistant entomologist, North Carolina Department of Agriculture, Raleigh, for opportunity to examine the specimens and use the records at the State Department of Agriculture. I wish to thank, also, Dr. H. E. Ewing, of the Bureau of Entomology, United States Department of Agriculture, Washington, D. C., not only for permission to use records and to examine type specimens and other material in the United States National Museum in Washington, D. C., but also for assistance and helpful discussion. To Professor J. P. Givler, in charge of the biology department, Woman's College, University of North Carolina, and to Dr. E. A. Andrews, Professor Emeritus of Zoölogy, of the Johns Hopkins University, I am grateful for valuable suggestions.

I have found, in North Carolina, eleven different species of fleas representing ten genera. These fleas have been taken chiefly from ten common hosts, including cottontail rabbit (*Sylvilagus floridanus mallurus*), gray squirrel (*Sciurus carolinensis carolinensis*), flying squirrel (*Glaucomys volans volans*), opossum (*Didelphis virginiana*), wharf rat (*Rattus norvegicus*), house mouse (*Mus musculus*), common mole (*Scalopus aquaticus*), house cat (*Felis domestica*), dog (*Canis familiaris*), and chicken (*Gallus domesticus*), the collections coming from 67 of the one hundred counties of this state. An examination of the specimens in the North Carolina Department of Agriculture, at Raleigh, and in the United States National Museum, at Washington, D. C., shows that one of these eleven species has apparently not been previously reported from North Carolina, and that two additional species have been recorded from this state. The entire list follows.

Order SIPHONAPTERA

Sticktight or Tropical Chicken Flea—*Echidnophaga gallinacea* Westwood (1875)

Previous records: The records at the North Carolina State Department of Agriculture include specimens from Wilmington, New Hanover County, from chickens and dogs; Brunswick County, from chickens; Clarkton, Bladen County, chickens; Wallace, Duplin County, chickens; Conetoe, Edgecombe County, host not stated; Raleigh, Wake County, kitten and wharf rat; Troy, Montgomery County, chickens; and Henrietta, Rutherford County, from chickens. The United States National Museum has records of specimens from dog, collected June 15, 1931, at Wilmington, by W. E. Merritt.

Present records: I have examined 38 North Carolina collections of this species, which included 995 specimens from 29 counties, distributed as shown in Table I. Their distribution according to hosts was as follows: house cat 497, dog 469, chicken 29.

It is claimed that this species was originally confined to the old world, but has been introduced into tropical America. It has been reported in southern United States, including Texas, New Mexico, and California, and, in 1927, as far north as Cape Henry, Virginia, where, according to records from specimens in the Bureau of Entomology at the United States National Museum, it was reported from chicken. Jordan (1929c) lists a record of specimens taken as far north as New York City, from rat. Some other National Museum records for this species include such varied hosts as Cooper's hawk, rat, coyote, young quail, ground squirrel, mongoose (Hawaii), and horse (Orangeburg, S. C., Nov. 1894).

An examination of the map, Fig. 1, showing the distribution of the records of the sticktight flea from the various counties of North Carolina, indicates that this species is limited to the eastern and southern portions of the state, although collections of fleas have been made in many of the counties farther west, including rather large collections in Guilford and Wilkes counties. This apparently restricted distribution extends somewhat beyond the limits of the coastal plain region of the state, and rather closely approximates the extent of the lower austral life zone which was outlined by C. S. Brimley in 1913, and later discussed by Pearson, Brimley, and Brimley (1919) in their book on the birds of North Carolina. A study of some of the factors influencing the

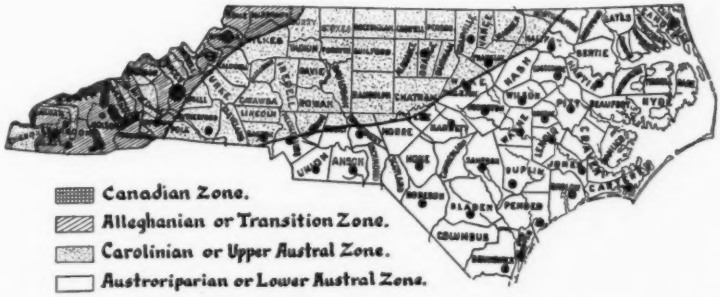

FIG. 1.—Map of North Carolina showing the distribution, by counties, of the sticktight flea, *Echidnophaga gallinacea*, from records (based upon specimens) in the North Carolina Department of Agriculture and in the personal collection of A. D. Shaftesbury. (Base map of Life Zones from "Birds of North Carolina" by Pearson, Brimley, and Brimley, used by permission of J. W. Harrelson, Director N. C. Dept. Conservation and Development.)

permanent and perhaps seasonal distribution of *E. gallinacea* might yield interesting results. Is this species gradually spreading toward the north and west, or are there factors which limit the range more or less to what is apparently its present distribution? From the data shown in Table I it will be seen that the few counties which show large collections of this species are well toward the southeastern coast line of the state.

Oriental Rat Flea, Indian Plague Flea—*Xenopsylla cheopis* Rothsch. (1903)

Previous records: State Department of Agriculture—from cotton rat (*Sigmodon hispidus* Say & Ord.), at Raleigh, determined July 29, 1910, by N. C. Rothschild.

Present records: From *Rattus norvegicus*—5 specimens collected Nov. 6, 1931, Raleigh, Wake County; 1 specimen, Jan. 5, 1932, from Greensboro, Guilford County; 1 specimen, Mar. 17, 1932, Greensboro; 3 specimens, Apr. 19, 1932, Greensboro; from *Mus musculus*—2 specimens, Nov. 26, 1932, Raleigh; 1 specimen, Aug. 7, 1933, Plymouth, Washington County.

It is claimed that this is the flea chiefly concerned with the transmission of bubonic plague in the old world, and, according to Fox (1925, p. 133), it "is cosmopolitan in its distribution and is present on the East, South and West coast of the United States. Whether it is present in the interior of the United States is not known." The North Carolina records of this species, though few, offer two points of interest, (1) they are, with one exception, from counties well inland, and (2) a part of them are from the house mouse, which has yielded but few records of any species of fleas.

Human flea—*Pulex irritans* Linnaeus (1758)

Previous records: The only record at the State Department of Agriculture was from a deserted house in Buncombe County; records at the United States National Museum include specimens from West Raleigh, Wake County, Apr. 8, 1915, and Halifax County, collected by E. B. Marshall, Aug. 14, 1931.

Present records: I have examined 69 collections of this species from North Carolina, including 1228 specimens from 52 counties, as listed in Table I. The distribution of hosts was as follows: dog 1169, man 39, domestic rabbit (*Lepus* sp.) 8, cottontail rabbit 8, cat 3, gray squirrel 1.

This European species is reported as having a restricted distribution in the United States. Ewing (1929) stated that it was "found particularly in California but occurs sparingly in the Mississippi valley." Later (1931) the same writer mentions the apparently uneven distribution of *Pulex irritans*, also mentioned by Jordan (1929a), and gives a map of the United States showing this irregular distribution, stating that "certain areas exist in which repeated search for this flea has failed to reveal its presence." Dr. Ewing (1931, p. 366) suggests that the absence of records from the southern Appalachian region is probably due to lack of collecting. He recalls that Jordan (1929a) predicted that *P. irritans* would be found in this section. Regarding the coastal plain region, however, Dr. Ewing says that "in his extended work over a period of several years in the eastern parts of the states of Maryland, Virginia, and North Carolina he has never taken a specimen of *Pulex irritans*."

As has been mentioned, I have examined over 1200 specimens of *P. irritans* from 52 North Carolina counties, ranging from Clay and Jackson counties in the west, to Carteret and Currituck counties on the east coast, and, although it varies considerably in abundance in different localities, this species is apparently just as widespread in North Carolina as the cat and dog fleas (*Ctenocephalides felis* and *C. canis*). *At present*, it could probably be found in any settled section of this state. In this connection, it might be significant to recall my experience with this species in Guilford County. During the years 1924 to 1929, only an occasional specimen of *P. irritans* was found among the 40 or 50 specimens of fleas collected each year by the zoölogy students from cats and dogs about the campus of Woman's College. Since 1929, however,

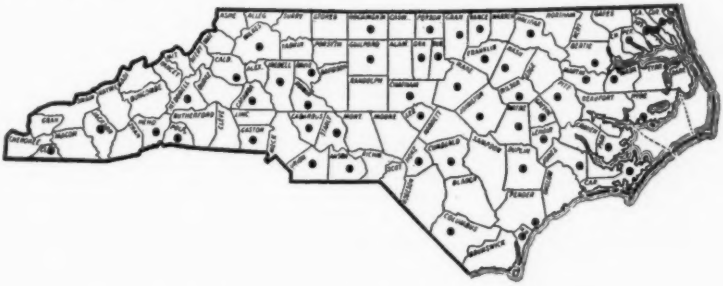

FIG. 2.—Map of North Carolina showing the distribution, by counties, of the so-called human flea, *Pulex irritans*, as indicated by the records of specimens in the writer's collection.

Pulex specimens have been present in much larger numbers. From this, it seems possible that *P. irritans* has appeared in the northeastern coastal plain region of the state since Dr. Ewing's field work in that locality, although it must be admitted that Dr. Ewing's collections were from wild animals, and there would probably be little chance of finding a specimen of *Pulex irritans* on them except an occasional accidental stray, since it is a species which infests domestic animals.

Hoplopsyllus (Baker, 1905) sp.

Previous records: Specimens of fleas taken from opossum in Raleigh, Wake County, Jan. 10, 1921, by C. S. Brimley and W. B. Mabee, were identified by them as belonging to this genus.

These specimens are now in Mr. Mabee's possession, in Montana, and I have not had an opportunity to examine them. No special opossum flea is known. My own collections from opossums have yielded only a few specimens of the squirrel flea, *Orchopeas wickhami*, and of the cat flea, *Ctenocephalides felis*.

Dog flea—*Ctenocephalides canis* Curtis (1826)

Previous records: A number of records at the North Carolina Department of Agriculture, from scattered counties and various hosts.

Present records: My records for this species include 76 collections from North Carolina, with 863 specimens from 51 counties, as listed in Table I. Distribution of hosts was as follows: dog 804, cat 9, domestic rabbit 1, "dogs and cats" 49.

Cat flea—*Ctenocephalides felis* Bouché (1835)

Previous records: A few in the State Department of Agriculture, of specimens taken at Raleigh and Wilmington, from opossum and dog.

Present records: My records include 74 collections, with 2182 specimens from 49 counties, as shown in Table I. Distribution according to hosts was as follows: house cat 386, dog 1671, opossum 4, man 3, cottontail rabbit 1, flying squirrel 1, "cats and dogs" 31, host not recorded 85.

Although Baker (1904, p. 385) objected to Rothschild's differentiation of *canis* and *felis*, this distinction is now universally accepted. My records show fewer *C. canis* than *C. felis*, although many more dogs than cats were examined. These two species of fleas are apparently found throughout North Carolina, and, though there is considerable difference in their proportional abundance in different localities, *C. felis* is apparently more abundant in most localities, particularly in the eastern part of the state. Both are species which infest domestic hosts, and only an occasional accidental stray specimen would be expected from wild animal hosts. Some of my collection data suggest the possibility of a seasonal difference in the proportions of *C. felis* and *C. canis*, with the former species relatively more abundant during the warmer months of the year.

Rabbit flea—*Cediopsylla* (*Spilopsyllus*) *simplex* Baker (1895)

Previous records: Wilmington, New Hanover County, collected from rabbit, by M. Kisliuk, Dec. 22, 1920. (N. C. Dept. Agri.)

Present records: My records for North Carolina include 12 collections, with a total of 189 fleas from 8 counties, as follows—Caldwell 1, Guilford 47, Harnett 1, Mecklenburg 1, Orange 20, Transylvania 1,

TABLE I

THE 1930–33 COLLECTIONS OF THE FOUR MOST COMMON SPECIES OF FLEAS IN NORTH CAROLINA, LISTED BY COUNTIES

COUNTY	CTENOCEPHALIDES CANIS	CTENOCEPHALIDES FELIS	PULEX IRRITANS	ECHIDNOPHAGA GALLINACEA	COUNTY	CTENOCEPHALIDES CANIS	CTENOCEPHALIDES FELIS	PULEX IRRITANS	ECHIDNOPHAGA GALLINACEA
Anson		61	3	2	Jones	12	123	82	62
Bertie	1	96	1		Lee		135	3	9
Brunswick		18		215	Lenoir	1	2	9	338
Buncombe	39	30			Lincoln	2	30		
Burke	3	40			McDowell	5		5	
Cabarrus	15	25	20	6	Martin	30	104	31	1
Caldwell	8	47	3		Mecklenburg		62		5
Camden			2		Nash	11	9	31	
Carteret	26	9	42	2	New Hanover		254	5	10
Catawba	12	14	4		Onslow		1		6
Chatham	1		1		Orange	10	1	10	8
Cherokee	2	14			Pamlico	52	8	67	
Clay	3	3	50		Pasquotank	5	1	1	
Columbus		23	14		Pender		96	10	107
Cumberland	1	30	1		Perquimans	3	20	2	
Currituck	192	73	134	2	Person	11	21	11	
Davie			61		Pitt	6		6	10
Duplin	1		2		Polk	2		4	
Durham	6	1	1		Robeson	2			1
Franklin	3		40	1	Rockingham	8	9	12	
Gaston	4	27	9	2	Rowan	22		3	
Granville	4	25	2	4	Sampson				4
Greene	1	39	14	3	Stanly	1	19	1	
Guilford	116	315	92		Surry	30	2		
Halifax	33	4	15	11	Union	1	9	2	18
Harnett	3	1		24	Vance	28	19	2	
Henderson	3	4	24	2	Wake	4	1	1	
Hoke	1		1	46	Washington	8	33	4	
Hyde		148	20		Wayne	6	98	4	9
Iredell	18	4	37		Wilkes	92	33	325	
Jackson	1	41	1		Wilson			2	10
Johnston	1		1	77	Yadkin	13			
					Totals	863	2,182	1,228	995

Wilkes 33, and Yadkin 85. Distribution according to hosts: cottontail rabbit 153, dog 35, house cat 1.

This is apparently the common rabbit flea of this region, although more extensive collections might prove otherwise. The United States National Museum has records from rabbits in Michigan, Iowa, Massachusetts, Georgia, and Tennessee. I did not find it among a small collection of fleas which I made from cottontail rabbits (*Sylvilagus floridanus?*) and jack rabbits (*Lepus californicus melanotis*) in Stafford County, Kansas, in December 1931.

Mole Flea—*Ctenophthalmus pseudagyrtes* Baker (1904)

Previous records: Some records in the State Department of Agriculture, Raleigh, taken at Raleigh from *Scalopus aquaticus*; a U. S. N. M. record, from meadow mouse, *Microtus* sp., collected by R. L. Boke, on Oconalufty Creek, Swain County, Apr. 18, 1931.

Present records: All from *Scalopus aquaticus*—5 specimens collected July 24, 1932, in Granville County; 3 specimens, Sept. 19, 1932, Guilford County; and 1 specimen, Mar. 30, 1933, Guilford County.

The National Museum specimen records of this species are not very abundant and are from the eastern and central states, chiefly from various species of moles, shrews, and pocket gophers. It will likely be found in the state wherever suitable hosts are found.

Rabbit Flea—*Odontopsyllus multispinosus* Baker (1904)

Previous records: From *Sylvilagus floridanus mallurus*, collected Dec. 7, 1899, at Raleigh, by H. H. Brimley and C. S. Brimley.

Present records: All from cottontail rabbit—3 specimens collected Apr. 6, 1931, Guilford County; 7 specimens, Apr. 13, 1931, Yadkin County; 2 specimens, Dec. 30, 1931, Guilford County; 2 specimens, Apr. 16, 1932, Orange County; and 2 specimens, Apr. 25, 1932, Guilford County.

This species was originally described from one male specimen captured at Raleigh. The few specimens recorded at the National Museum are from North Carolina, Maryland, and Massachusetts, and all are from varieties of cottontail rabbits. Many of the live and freshly killed cottontail rabbits which I have examined in North Carolina have had no fleas of any kind. The fact that, during our collections, 153 specimens of *Cediopsylla simplex* have been taken from cottontail rabbits, while only 16 specimens of *O. multispinosus* have been secured, suggests that this latter species might be relatively more abundant on some other kind of rabbit in some other locality.

Squirrel flea—*Orchopeas wickhami* Baker (1895)

Previous records: One specimen, from flying squirrel, Raleigh, Wake County, Jan. 25, 1927. (N. C. Dept. Agri.)

Present records: I have examined 18 collections from North Carolina, with 370 specimens from 11 counties, distributed as follows—Caswell 11 specimens, Chatham 46, Forsyth 2, Guilford 225, Mecklenburg 3, Onslow 34, Orange 8, Rockingham 12, Union 25, Wake 2, and Wilkes 2. The distribution according to hosts is as follows: gray squirrel 353, flying squirrel 8, opossum 6, and dog 3.

This species, recorded chiefly from squirrels and flying squirrels, has been reported from various of the eastern and central states, and as far west as Arizona. It has been introduced into England with the gray squirrel (Jordan, 1928). It seems likely that it will be found throughout North Carolina, wherever suitable hosts are found. Further collections are needed, particularly in the eastern and western sections of this state.

Orchopeas leucopus Baker (1904)

This species, which is closely related to the preceding, has been reported to the National Museum by R. L. Boke from specimen taken in 1931 from the white footed deer mouse, *Peromyscus leucopus leucopus*, on Oconalufty River, Swain County, at an altitude of 3,000 feet.

European and North American Rat Flea—*Nosopsyllus fasciatus* Bosc. (1801)

Previous records from North Carolina: None.

Present records: From *Rattus norvegicus*—1 specimen collected at Greensboro, Guilford County, Mar. 27, 1931; 1 specimen collected at Greensboro, Mar. 16, 1932, by E. E. Brown; 3 specimens from Winston-Salem, Forsyth County, from a rat sent to Greensboro, Apr. 13, 1932, by A. E. Oman, leader, rodent control, U. S. Biol. Survey; 4 specimens collected at Greensboro, Oct. 26, 1932 (from rats infected with a protozoan blood parasite, *Trypanosoma lewisi*); and 3 specimens collected by Miss R. Collie, Raleigh, Feb. 15, 1933 (courtesy C. S. Brimley, N. C. Dept. Agr.)

Interest in the study of the incidence of rat fleas has been added by the recent increase in the number of cases of typhus fever in the southern states. Our endemic typhus is not transmitted by the body louse ("cootie"—*Pediculus humanus*) as is the European typhus. Since the virus of endemic typhus has been recovered from *Xenopsylla cheopis* taken from rats trapped at typhus fever foci, in Baltimore and Savannah, and both *X. cheopis* and *Nosopsyllus fasciatus* readily transmit the

typhus virus from infected to noninfected white rats, they are strongly suspected as being among the agents of transmission of endemic typhus from rats to man. (Cf. Dyer, Ceder, Rumreich, and Badger, 1931; Dyer, *et al.*, 1932.)

Mouse flea—*Ctenopsyllus segnis* Schönherr (1816) (= *Leptopsylla musculi* Duges, 1832)

Previous records: Specimens taken from cotton rat, *Sigmodon hispidus*, at Raleigh, were determined by N. C. Rothschild, July 29, 1910; other specimens, taken from wharf rat at Raleigh, August 1911, were determined by W. B. Mabee and C. S. Brimley.

Present records: Two specimens collected from house mouse at Plymouth, Washington County, Aug. 7, 1933.

Following is a list of the thirteen species of fleas which have been reported from North Carolina: *Echidnophaga gallinacea**, *Xenopsylla cheopis**, *Pulex irritans**, *Hoplopsyllus* sp., *Ctenocephalides canis**, *Ctenocephalides felis**, *Cediopsylla simplex*, *Ctenophthalmus pseudagyrtes*, *Odontopsyllus multispinosus*, *Orchopeas wickhami*, *Orchopeas leucopus*, *Nosopsyllus fasciatus**, and *Ctenopsyllus segnis**. I have collected and examined specimens of eleven of these species, including all except *Hoplopsyllus* sp. and *Orchopeas leucopus*. These two were added from specimen records at the North Carolina Department of Agriculture, and at the United States National Museum, at Washington, D. C. *Nosopsyllus fasciatus* has apparently not been previously reported from North Carolina. The seven species which are starred (*) are not native to this section, but are introduced species, and include those most annoying to man and domestic animals, and those most likely to transmit disease.

Jordan (1929a) estimated in 1928 that there were about 800 known species of fleas, of which 131 were known in United States and Canada, the larger number of the North American species being from the western United States. A recent examination which I made of flea specimens in the United States Department of Agriculture, at the National Museum, revealed the fact that there were fewer flea records from North Carolina than from almost any other state. Thirty-one species have been listed from New York (Jordan, 1929c), but, as far as I know, no such definite data are available regarding the fleas of any other eastern state. It should not be inferred that the accompanying list of thirteen species represents anything like the entire number of species of fleas in North Carolina. This list likely represents less than a fourth of the species that should be found in this state.

Other species of fleas which have been collected in neighboring states

and might be expected in North Carolina include *Ceratophyllus riparius*, from nest of bank swallow (*Riparia riparia*) at Roslyn, Va., collected June 1916, by F. C. Bishopp (record from Jordan & Rothschild, 1920); *Stenoponia wetmorei*, collected from a white footed mouse, *Peromyscus leucopus*, in 1927, at Falls Church, Va. (record from U. S. N. M.); *Ctenopsyllus* (*Leptopsylla*) *catatina*, described by Jordan (1928) from a specimen taken from an opossum in Pennsylvania; *Neopsylla wenmanni* Rothsch. (1904), collected by R. L. Boke, April 1931, from white footed mouse, *Peromyscus leucopus leucopus*, at an altitude of 3,000 ft., at Greenbriar, Tenn. (U. S. N. M.); and *Ctenopsyllus* (*Leptopsylla*) *selenis* Rothsch., collected from white footed mouse, *Peromyscus maniculatus*, at Buttry's Cave, Jefferson City, Tenn., Feb. 4, 1933 (U. S. N. M.). *Rhopalopsyllus gwyni*, which was described from fleas of both sexes taken from "rats" at the quarantine station at Brunswick, Ga., in 1904, was believed by Fox (1914) to have come from South America, but numerous specimens of a very similar, perhaps identical, species, *Rhopalopsyllus sigmodoni*, were collected at Houston, Texas (Stewart, 1930), chiefly from *Rattus norvegicus* and the cotton rat, *Sigmodon hispidus*. These records suggest that if *Rhopalopsyllus* is not a native genus, it has perhaps become established in some localities in North America and might be expected from cotton rats and other rats in North Carolina.

The mountain regions of North Carolina have not been examined at all for ectoparasites. Collections from chickarees, fox squirrels, and marsh rabbits have not been made, and examination of such forms as shrews, field mice, and bats would doubtless reveal several species unrecorded for this section, and possibly some new species. A careful examination of the nests of both domestic fowl and wild birds, which has not been made in North Carolina or elsewhere in the Americas, will no doubt yield specimens of both European and American species of bird fleas. Only seven species of Ceratophylli infesting North American birds were listed in 1920 by Jordan and Rothschild. Jordan (1929b) has listed 26 species of North American birds from which fleas have been taken. His own specimens of bird fleas from the United States were taken mainly from the nests of birds in Massachusetts and New Hampshire, and included but few new species. The nests which have yielded fleas in United States include not only some of ocean birds but several of common birds, such as English sparrow, ovenbird, cat bird, wood thrush, robin, and blue bird, so it seems likely that an examination of recently deserted bird nests in North Carolina should reveal at least a few species of bird fleas. A study of the distribution and

predominance of the two species of rabbit fleas throughout the state should be of interest, and a similar study of *Xenopsylla cheopis* and *Nosopsyllus fasciatus*, and other species which might be found on rats should be of value from the point of view of public health.

SEX RATIOS

The accompanying list (Table II) includes all of the specimens of fleas which I have identified from North Carolina, tabulated according to species and sex. From this table it will be seen that with the six species which have been collected in considerable numbers, there is a decided preponderance of females. The range in variation is from 62.2 per cent females in *Pulex irritans*, to 86.6 per cent females in *Echidnophaga gallinacea*, or, expressed in proportions, from 1 male to 1.6 females in *P. irritans*, to 1 male to 6.4 females in *E. gallinacea*. These figures represent, of course, the sex ratios of collected adult fleas.

TABLE II

SEX RATIOS OF FLEAS COLLECTED IN NORTH CAROLINA, 1930–33

	♂	♀	TOTAL	MALES PER 100 FEMALES
Ctenocephalides felis	626	1,556	2,182	40.2
Pulex irritans	463	765	1,228	60.5
Echidnophaga gallinacea	133	862	995	15 1
Ctenocephalides canis	295	568	863	51.9
Orchopeas wickhami	110	260	370	42.3
Cediopsylla simplex	68	121	189	56.1
Odontopsyllus multispinosus	3	13	16	
Xenopsylla cheopis	7	6	13	
Nosopsyllus fasciatus	3	9	12	
Ctenophthalmus pseudagyrtes	1	8	9	
Ctenopsyllus segnis	0	2	2	

Herms (1923), in tables showing the interchange of hosts and predominance of species, gives figures showing the number of each sex collected for several species of fleas. From California ground squirrels, he collected 2065 males and 2306 females of *Ceratophyllus acutus*, and 86 males and 140 females of *Hoplopsyllus anomalus*. The former gives a ratio of 89.5 males to 100 females, while the sex ratio in the latter species is 61.4 males per 100 females. Herms collected 117 males and 220 females of *Pulex irritans*, from humans, the ratio in this case being 53.1 males per 100 females. To a part of his Table XV (l. c., p. 326), which

he adapted from McCoy (1909, p. 1014), showing the predominance of species of fleas of the brown rat, *Rattus norvegicus*, in California, I have added the sex ratios, with the results shown in Table III.

The ratios shown in Table III agree, in a general way, with the sex ratios which I have found with fleas from various localities in North Carolina. However, Kopstein (1932) collected 113 male and 79 female specimens of *Xenopsylla cheopis*, from rats in the harbor warehouses of Tjilatjap, Java. This gives a sex ratio of 143.0 males per 100 females, but the proportions in his collections in other localities in Java differed from this, so that the total of all of his listed collections of *X. cheopis* (l. c., pp. 413, 421–422, 426), from Java, was 555 males and 672 females, which gives a ratio of 82.5 males per 100 females. His collections of this species from mountain localities of West Java (l. c., p. 426) alone totalled 341 males and 466 females, which gives a ratio of 73.1 males per

TABLE III

SEX RATIOS OF FLEAS FROM BROWN RATS IN CALIFORNIA (MODIFIED FROM McCOY, 1909)

	♂	♀	MALES PER 100 FEMALES
Nosopsyllus fasciatus	570	1,252	45.5
Xenopsylla cheopis	790	1,146	68.9
Pulex irritans	225	425	52.9
Ctenopsyllus segnis	44	137	32.1

100 females. Kopstein's smaller collections of two other species were: *Xenopsylla astia*, males 76, females 103; *Stivalius cognatus*, males 165, females 219. These give ratios of 73.7 and 75.3, respectively.

These data appear to indicate that the sex ratio of collected adult fleas is commonly below 80 males per 100 females, and, in fact, in most cases, is around 60, or less, males per 100 females.

Sex ratios which differ considerably are found in other animals. Gudger (1906) found a ratio of 3 males to 7 females in *Siphostoma floridae*, a pipefish at Beaufort, North Carolina. Hildebrand (1927), with 103,150 adult top minnows, *Gambusia*, collected in the Beaufort region from June to December, found a decided seasonal variation in the proportions of the sexes, though the females were always predominant, the average ratio being 1 male to 4.4 females. Hildebrand (1933) found among 1442 mature Carolina diamond-back terrapins (*Malaclemmys centrata*) in captivity a ratio of 1 male to 5.9 females. Hill (1926)

with *Platygaster hiemalis* Forbes, a hymenopterous parasite of the Hessian fly, found that approximately 66 per cent of the adults were females. Gershenson (1928) found in the dipterous insect, *Drosophila obscura*, a sex linked factor which caused an abnormal sex ratio of 96 per cent females. These are fairly typical of many records, and apparently in most cases the females predominate. Often the difference is due to a differential birth rate, as is the case with such insects as aphids, ants, and honeybees, where the females, though many are sterile in some of these forms, are strikingly predominant, due to an actually controlled sex ratio of births.

I have been unable to distinguish the sex of flea eggs, and, with the rather limited number of larvae which I have examined, I could not be certain of the species, much less the sex. Careful examination of a sufficient quantity of material would likely obviate this difficulty, but the question would be only partially settled until the effects of temperature, season and weather, kind and amount of food, hybridization, and other factors, which affect markedly the sex ratios in other organisms, have been considered.

There might be a selective mortality, as occurs with many other animals, which might later alter markedly the sex ratios shown at birth. There is a popularly held opinion, which has often been put into print (Geiser, 1923, p. 161; Popenoe, 1926; Wells, Huxley, Wells, 1931, pp. 555-ff.), that in many different groups of animals the males are less viable than the females, that the male is the weaker sex. Very little is known regarding the comparative length of life of the males and females of even the common insects. Hirst (1926), working on the transmission of plague by rat fleas, found that in *Xenopsylla cheopis* and *X. astia* the females live much longer when unfed than do the males, and that a heavy growth of *Bacillus pestis* in the proventriculus causes death in the males much earlier than in the females. With flies of the family Tabanidae, C. S. Brimley informs me, the sex ratios are approximately equal at emergence, but if these flies are caught as they are found in nature, they will give a ratio of many females to one male. With the much investigated common fruit fly, *Drosophila melanogaster*, the sexes at emergence from the pupal stage are about equal in numbers, but Pearl (1928) has shown that on the average the females live longer than the males. (Cf. also, Alpatov and Pearl, 1929.) Female fleas have apparently not reached the high state of economic efficiency of the praying mantis (*Mantis* sp.), which devour the males after mating. The mating of fleas, with the males beneath, has been observed several times, and

Lundblad (1927) noticed the use of the (larger) antennae of the male as clasping organs, but none of the observers has suggested that the male may die as a result of the mating act. Incidentally, does the male flea ever mate with more than one female? If he mated only once, he would be useless thereafter, and might die before the female. What is usual in this regard, with the males of other insects?

Possibly there is no real differential sex ratio, merely an apparent one due to some physical or physiological difference between the sexes. Perhaps the females are required to spend more time on the host in order to provide for the larger amount of nourishment necessary to develop the eggs. Leeson (1932), working on the sex of newly emerged rat fleas (*X. cheopis*), found that all of the females emerged before the males began to emerge. If this is true with other species, then it seems that fleas collected during the beginning of a period of emergence would show a preponderance of females. It has been suggested that the female fleas are larger and are more easily seen, or perhaps they are more sluggish and are more easily captured. Careful dusting and combing of numerous host animals has yielded similar ratios, and has even resulted in the capture of numerous mites smaller than the smallest male fleas. All of which suggests that considerable further investigation must be done before the solution of the riddle of the apparent preponderance of females in the collections of fleas of North Carolina, and other localities, can be even approximated.

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NEARLY RELATED COPEPODS DIFFERENTIATED PHYSIOLOGICALLY AS WELL AS MORPHOLOGICALLY[1]

(*Cyclops vernalis* Fischer, *C. venustoides*, n. sp., and *C. exilis*, n. sp.)

By R. E. Coker

Seven Text-figures

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INTRODUCTION

Freshwater cyclopid copepods are notable in that usually the diagnostic characters are relatively easily observed (as compared with those of harpacticoids, for example) and susceptible of rather precise statement. Nevertheless, in the old major group (Schmeil) of *bicuspidatus-viridis-vernalis*, there occur in various parts of the world so many forms marked by seemingly very small but yet clearly definable differences that much doubt and difference of opinion have properly prevailed as to whether in many cases we have to do with distinct species, or with widely occurring ecological variations or mutations. Such copepods seem to lend themselves well to experimental breeding, and we were particularly prompted to engage in such experiments by discovering at Chapel Hill three forms living in close association and evidently of the same general type, although clearly and immediately distinguishable

[1] Contribution from the Laboratory of Zoology, University of North Carolina, and the Laboratoire d'Evolution, University of Paris. Part of an investigation of copepods aided by grant from the Rockefeller Fund for Research in Pure Science at the University of North Carolina.

by the numbers of segments in the first antennae. Some results of the breeding experiments are treated primarily in other places (Coker, 1933, etc.); but, as a basis for the appraisal of experimental results, it is essential to give adequate taxonomic definition to the types used, and that is the purpose of the present paper. We are able to report notable physiological as well as morphological distinctions.

The copepods in question have, respectively, 17, 12, and 11 segments in the first antennae. Those with 12 and 11 segments are discussed later in this paper. The copepod with 17-segmented antenna we must identify either as *Cyclops vernalis* Fischer or as *C. robustus* Sars, but, by reason of our own observations to be given later, as well as those of Lowndes (1929), we are compelled to place *robustus* in the synonomy of *vernalis*.

Cyclops vernalis Fischer

Cyclops vernalis Fischer 1853
Cyclops elongatus Claus 1863
Cyclops lucidulus Sars 1863
Cyclops robustus Sars 1863
Cyclops parcus Herrick 1882
Cyclops brevispinosus Herrick 1884
Cyclops americanus Marsh 1893

Fischer's *vernalis* was twice redescribed under different names before Schmeil, in his classical monograph of 1893, again redescribed it and identified with it Claus's *elongatus*, having 18-jointed antenna, and with the copepod described by Sars as Koch's *lucidulus*. After Schmeil the species has been well-recognized by students of copepods of Europe, Asia, and North Africa. It seems not to have been recorded as such by American authors, but Sars (1918) placed Herrick's *parcus* in the synonomy of *lucidulus* and Kiefer (1929), rightly we believe, returned *lucidulus*, and *parcus* with it, to the synonomy of *vernalis*. Kiefer also assigned Marsh's *americanus* to the synonomy of Sars's *robustus*, as Lilljeborg (1901) had already done with Herrick's *brevispinosus*, while Lowndes has correctly relegated *robustus* to the synonomy of *vernalis*.

Our copepods conform so closely with *vernalis* in all essential particulars that no extended description need be given. It may be mentioned that we have studied *vernalis* of the vicinity of Paris and have bred them for several generations in the laboratory, so that we are able to make helpful comparisons between the American and European copepods. The accompanying figure of the whole copepod (fig. 1 a) shows the characteristic form of the body, the short 17- or 18-jointed

antenna (17 segments in the right and 18 in the left antenna of the example figure), not longer than the first segment of the body, the outwardly produced postero-lateral angles of the dorsa of the last two thoracic segments (the form of the next to last being particularly char-

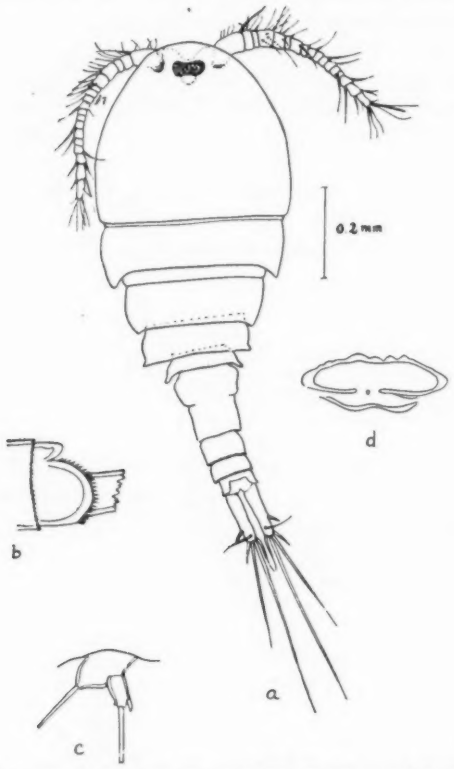

FIG. 1. *Cyclops vernalis* Fischer, Chapel Hill, N. C.: *a*, adult female with 17-jointed antenna on right side and 18-jointed antenna on left side; *b*, last abdominal segment and anal plate; *c*, fifth foot; *d*, seminal receptacle.

acteristic, since many species have the last thoracic segment produced laterally), the prominent lateral lobes of the genital segment and the form of the furca. The seminal receptacle (fig. 1 d) conforms well with Schmeil's fig. 5 (Pl. II) and Kiefer's fig. 18. *Vernalis* seems to be the

only copepod that frequently has an 18-segmented antenna by division of the 7th segment, as in our example, or, more rarely, by division of the 4th.[2]

The spine formula (for terminal segments of exopodites of P_{1-4}) of *vernalis* is supposed to be 2–3–3–3, that of *robustus* 3–4–4–4. In our examples from Chapel Hill the formula is almost always 3–4–4–4 in females, both wild and bred, and 2–3–3–3 in males bred from the same parents as the females with the other formula. Occasionally the reared males have formulas of 3–4–4–4, or 3–4–4–3 (on one or both sides). A few females reared from the same batch of eggs as those with the "typical" armature have the 2–3–3–3 formula, and one had the variant 2–4–4–4 (one side). The European copepods reared in Paris in a pure line starting with a female with formula 3–4–4–4 display that formula in only rather more than half the examples examined; quite a few have the formula 2–3–3–3, and a good many have such aberrant formulas as: $\frac{3\text{–}4\text{–}5\text{–}5}{2\text{–}3\text{–}3\text{–}3}$ $\frac{3\text{–}3\text{–}4\text{–}3}{3\text{–}4\text{–}4\text{–}4}$ $\frac{3\text{–}4\text{–}3\text{–}3}{3\text{–}4\text{–}4\text{–}4}$ $\frac{3\text{–}4\text{–}4\text{–}3}{2\text{–}3\text{–}4\text{–}3}$ (compare fig. 4, *a*, *b*, *c*). The 2–3–3–3 and 3–4–4–4 formulas may be given by females reared from one batch of eggs under similar conditions of temperature and culture media, but the higher formula is more common in copepods reared at low temperatures, while the lower formula is commonest at high temperatures.

Lowndes (1926, 1927, and 1929) has already shown, by breeding experiments, that the spine formula is so variable in some copepods as to be of slight value for diagnostic purposes, and our observations are confirmatory of this conclusion, although not conforming with his experience that in *americanus* (?) the two forms breed true without giving mixed formulas. Since the alleged specific distinction of *robustus* now rests almost exclusively on the spine formula and an ill-defined difference in the proportions of the basal segment of P_5 it is not apparent how the more spinous form can be recognized as a species, entailing the identification in two species of copepods bred from the same parents.

The fifth foot (fig. 1 *c*) is appropriate to *vernalis*, except that the proximal segment is rather wide, as is also true in the Paris copepods; generally it is hardly more than twice as wide as the distal segment. The latter is more slender than shown by Schmeil, but is virtually identical with the corresponding segment in Kiefer's fig. 18 (1929) for *vernalis*.

The proportions of the terminal segments of the rami of P_4 are fre-

[2] We have reared an example of *C. viridis* with 18-segmented antennae, 2 segments representing the normal 7th segment.

quently used for diagnostic purposes, and doubtless properly so within reasonable limits; but these segments show much diversity of form, even in copepods reared from the same batch of eggs and under virtually identical conditions. The end segment of the exopod (fig. 4 *a*) is commonly broad, being more than half as wide as long. The end segment of the endopod is narrow, but shows notable diversity in proportions. Fig. 2 illustrates two forms found at Chapel Hill, and fig. 3 three forms noted in copepods found or reared at Paris. The quotient of

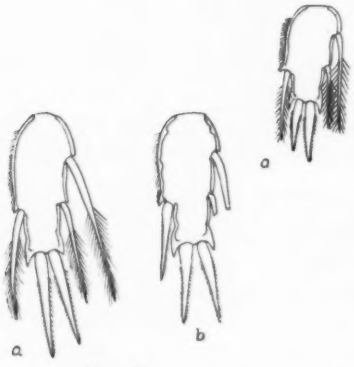

FIG. 2

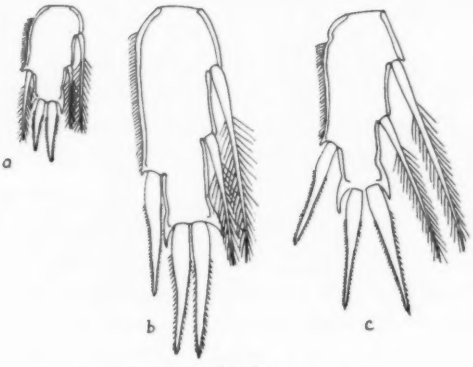

FIG. 3

FIG. 2. *Cyclops vernalis* Fischer, Chapel Hill, N. C., 4th foot, terminal segment of endopod: *a*, with seta on outer border; *b*, with spine on outer border.

FIG. 3. *Cyclops vernalis* Fischer, Paris, France, 4th foot, terminal segment of endopod: *a*, from a female (No. 1513) reared at high temperature, with seta on outer border; *b*, from wild female (No. 1), with spine on outer border; *c*, from a large female (No. 1606) reared at low temperature, with spine on outer border. Nos. 1513 and 1606 were progeny of No. 1 (second filial generation). *All drawn to same scale.*

length into breadth among the latter varied from 0.43 to 0.55 in females and from 0.39 to 0.52 in males. Quotients as diverse as 0.45 and 0.52 were obtained from copepods originating from the same batch of eggs and reared under essentially identical conditions. The outer border of this segment may be armed with a spine (fig. 2 *b* and 3 *b*, *c*) or with a seta (fig. 2 *a* and 3 *a*); whatever the armature of the parent, we have found the spine most commonly in copepods reared at low temperature, the seta in those reared at high temperature (Coker, 1934): this was true for the European copepods; we have not had the opportunity as

yet to make similar comparisons for the copepods at Chapel Hill. A spine in this place is one of the supposed distinctions of *Cyclops brevispinosus* Herrick. The 2 terminal spines of this segment are about equal in length, the mesial usually a little longer than the lateral, although sometimes the reverse is the case; the most extreme difference in size is illustrated in fig. 3 *a*, showing a rare condition.

Schmeil describes the posterior margins of the abdominal segments of *vernalis* as smooth; in our copepods, both reared and wild, at Chapel Hill and Paris, they are commonly inconspicuously dentate, sometimes notably so.

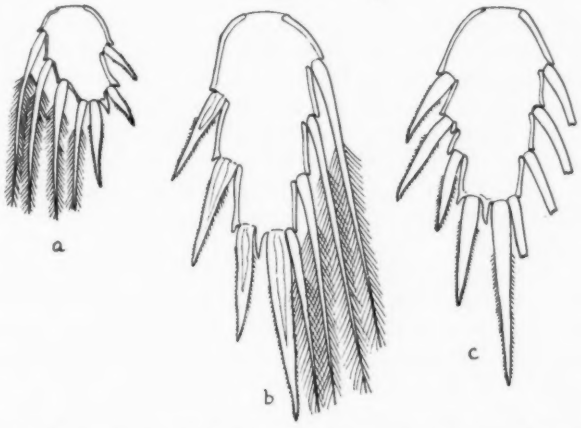

FIG. 4. *Cyclops vernalis* Fischer, Paris, France, 4th foot, terminal segment of exopod: *a*, from small female (No. 1513) reared at high temperature; *b*, from large wild female (No. 1); *c*, from large female (No. 164) reared at low temperature. Nos. 1513 and 164 were progeny of No. 1. *All drawn to same scale.*

Other figures are given as basis for comparison with the species next to be described. It should be noted that the anal operculum (fig. 1 *b*) is short, not reaching more than halfway to the posterior extremity of the segment and leaving the sinus largely open above. The furca may or may not show a distinct notch on the outer margin at the end of the basal third, and is without hairs on the inner margin.

Size: The length of *vernalis*, according to both Pesta and Kiefer is 1.2–1.7 mm., females, and 1.–1.2, males. We find that the size of the reared European copepod generally varies inversely with the tempera-

ture prevailing during the period of development, but this subject is treated in another paper. Wild examples taken from one locality at Chapel Hill in 1926, chiefly under ice, had lengths for females, 1.18–1.79, for males, 0.93–1.05 mm. Females taken from another locality during the winter of 1932 had lengths of 0.9–1.30 mm. Copepods reared from these at room temperature had lengths: of females, 0.84–1.09 mm., and of males 0.63–0.74 mm. The furcal rami varied in length from 8 to 14 per cent of the total length of the body, exclusive of the caudal setae (males and females). The rami are usually 4 or 5 times as long as the width at half length. The copepods of this species are generally smaller than those of the following species associated with them.

This copepod was readily bred for several generations in a culture medium consisting of a nearly pure culture of the alga Ankistrodesmus making a very green water. It was also bred in infusions of sheep manure or of hay, with Paramecium, Vorticella, Colpidium (?), and other Protozoa. One medium seemed to answer as well as another. Its development at room temperatures is very rapid, complete cycles from egg to egg being effected in several cases in the remarkably short period of 7 days, although sometimes as much as 10 days, occasionally more, was required. Except as to rate of development, the copepod seemed to be almost indifferent to temperature; the rate of development at about 7°C. was about 1/7 that at room temperature. Rates of development were essentially the same for copepods from Chapel Hill and from Paris. As is true of many other species, ovigerous females are often found under ice in winter.

Vernalis, although once called a stenothermal glacial relict species (Schokke, 1900), has long been known to be certainly eurythermal and a perennial breeder (Wolf, 1905; Pesta, 1928)— and this affords a point of contrast between it and the species described later in this paper.

Synonomy of *vernalis*, with special reference to *robustus* Sars, *parcus* and *brevispinosus* Herrick, and *americanus* Marsh

Schmeil dealt adequately with Claus's *elongatus* and Sars's re-identification of Koch's *lucidulus*. Sar's *robustus*, described 70 years ago, and redescribed and illustrated by the same author 55 years later, seems not to have been consistently interpreted. As described by Sars (1863 and 1918) it was not distinguished by the spine formula, for Sars recognized that *lucidulus* (= *vernalis*) might have either the lower or the higher formula; it was distinguished rather by the shortness and stoutness of the furca, by the spine on the outer margin of the end segment

of the endopod of the fourth foot and by a few very minor features. Recently, however, it seems to have been the practice to base the supposed distinctiveness of *robustus* primarily on the spine formula, notwithstanding that, if our experience indicates correctly, this would involve associating the name with examples having the furca relatively long and slender, and so doing violence to the original definition. As a matter of fact, the species is not well founded, as Lowndes (1929) has already shown. Lowndes reared typical *vernalis* from parents that were typically *robustus* in spinous armature and in other respects. His only disappointment was that, barring one exceptional case, he could not obtain the *robustus* form from either sort of parent. We have been able to supplement his work, not only by rearing both forms from the same parent, but also by showing that, to a considerable extent, the proportions of the furca, the chief distinction originally, is a function of the temperature at which the copepod is reared (Coker, 1934). It is possible that Lowndes's failure to obtain the *robustus* form in spinous armature from *robustus* parents was due to his care to rear the copepods at the constant temperature of 20°; while the higher spine formula may appear at that temperature, it seems much more likely to occur at low temperatures—7–12°.

Herrick's description of *parcus* (1884) and his figures make the near-identification of that species with *vernalis* inescapable. Why it should ever have been confused with *viridis*, as it was by Marsh (1906) and others, it would be difficult to say. The fifth foot is that of *vernalis*, not that of *viridis*, and the description of the seminal receptacle applies much better to the former species than to the latter, while the fact that the last two segments of the cephalothorax are described as "acute" should exclude *viridis* from further consideration. It may be remarked that, while *viridis* and *vernalis* are probably not distantly related, they are very different in general appearance and in behavior: *viridis* is a heavy-bodied species, relatively slow in movement and very slow and irregular in development; *vernalis*, on the other hand, is light-bodied, nervously active and extremely rapid in development, completing a life cycle under favorable conditions in about one-third the time required for *viridis*—one week, as compared with three weeks or more. Attempts to effect a cross between *viridis* and *vernalis*, either way, have been quite unsuccessful.

We know of no somatic characters to distinguish *parcus* from *vernalis*, but a difficulty remains to be considered. Chambers (1912) reported that American copepods he had identified as *viridis* had 12 chromosomes,

as does the European *viridis*, and that *americanus* had 10, and *parcus* 6. That *americanus* should have 10 chromosomes is as would be expected, for that is the number Braun (1909) found characteristic of *vernalis;* but that *parcus*, which is more typically *vernalis* than is *americanus*, should be ascribed a different number of chromosomes raises the question of identification. There is actually little in Chambers's characterization of the species to identify it with *parcus*. He does not mention the characteristic form of the posterior thoracic segments; he gives the spine formula, 2–3–3–3, which is applicable to many species; he describes the seminal receptacle as having a concave anterior margin and draws it deeply concave like that of *viridis*, which does not conform with Herrick's description—"oval," or with Marsh's "convex" (Marsh, 1920); the fifth foot as sketched is, however, like that of *parcus*. We have thus one positive character to identify Chambers's copepod with Herrick's *parcus*, and one serving the contrary purpose. Whether or not there is significance in the fact that the number of chromosomes found was exactly half of the number possessed by *viridis*, we do not know, but it seems probable that Chambers was dealing with representatives of an undescribed species, intermediate between *vernalis* and *viridis;* we have had some other reasons to suspect that such a species exists. In any event, the chromosome number can be of no taxonomic value except as it may be associated with distinctive somatic characters.

Cyclops brevispinosus was described by Herrick as a modified condition of *parcus*, the body and especially the caudal stylets more slender, the outer caudal seta reduced to a short ciliate thorn, the fourth foot modified by great enlargement of the spines and reduction of the setae, the number of the setae the same but "differently disposed," and the form of the seminal receptacle "slightly different;" the fifth foot, as figured, is that of *vernalis;* there seems to be absolutely nothing in the description to afford a basis of distinction from a species as variable as *vernalis* is now known to be. Lehmann (1903) showed that *brevispinosus* and *americanus* could not be positively distinguished. Marsh (1906) emphasized the supposedly distinctive character of the spine on the outer border of the terminal segment of the endopods of P_{2-4}, but that we find to be common, if not almost invariable, in *vernalis* when reared at low temperatures (Coker, 1934); Lilljeborg (1901) and Sars (1918) have already identified *brevispinosus* with *robustus*.

Among the examples I have reared, *vernalis*, as described by Sars, would seem to be best represented by most of the examples developing at intermediate temperatures, *robustus* of Sars by the occasional examples

developing at high intermediate temperatures that combine low-temperature form with high-temperature size; *americanus* by examples reared at low-temperatures (10°); *parcus* and Sars's unnamed "smaller variety having the caudal rami somewhat shorter" by copepods reared at temperatures above 25°.

We do not suppose that the last word has been said on the taxonomy of *vernalis*, and its varieties, if such they be, but we do feel that the perpetuation of names that correspond with no serviceable definitions renders no useful purpose—but rather the contrary, since the names are necessarily used without agreement as to their significance. We do not concern ourselves now with the 8 varieties of *vernalis* and *robustus* that Thallwitz (1926) has described and that seem to represent a few of the various modifications of spinous and setal armature, some, but not all, of which we have had appear in a single line along with more typical forms. Finally, it may be remarked that Lowndes (1926) has described and figured a form of seminal receptacle which he ascribes to *americanus*, and which he thinks distinguishes that species from *vernalis* and *robustus*; we do not know why the copepods possessing this form of seminal receptacle should be identified with *americanus*, but can express no further opinion regarding them.

Cyclops venustoides, n. sp.

A copepod found in several places at Chapel Hill, N. C., resembles *Cyclops vernalis* Fischer (1853) in general form but differs from it in several notable morphological characters. It is even more closely related to the comparatively rare *Cyclops venustus* Norman and Scott (1906), with which it may indeed be found to be identical, although there are distinct structural differences between it and that species. We have determined experimentally that there are important physiological distinctions between the copepod in question and *vernalis*, but we have not been able to make physiological comparisons with *venustus*. It may suffice to give figures and a brief structural description by comparison with *vernalis*, point out the few distinctions from *venustoides*, and mention the physiological peculiarities which have been amplified in another place (Coker, 1933).

The anterior portion of the body is smoothly oval, widest near the posterior margin of the long cephalic segment, and tapering uniformly to the last thoracic segment, which is hardly at all wider than the anterior portion of the following first abdominal segment (fig. 5 *a*). The last thoracic segment is short and markedly produced at the posterior

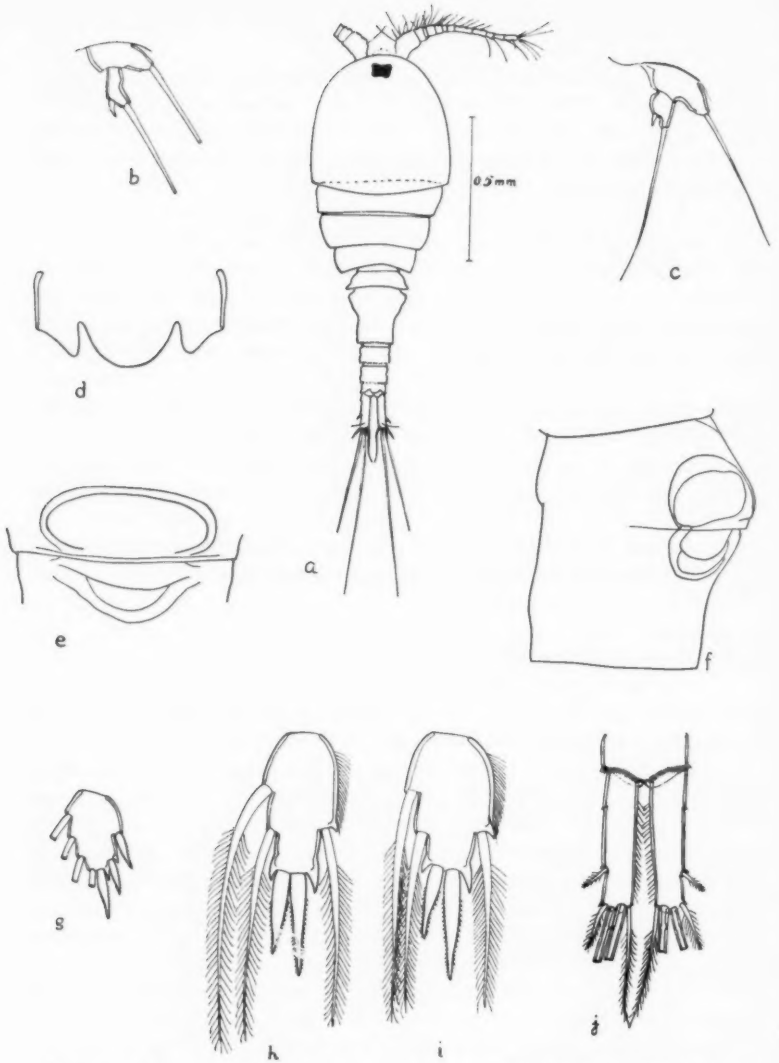

FIG. 5. *Cyclops venustoides* n. sp. (except *c*): *a*, adult female; *b*, fifth foot; *c*; 5th foot of *C. venustus*, Norman and Scott, for comparison; *d*, anal plate; *e*, *f*, seminal receptacle; *g*, 4th foot, exopod, terminal segment; *h*, *i*, 4th foot, endopod, terminal segment (more highly magnified); *j*, furca and last abdominal segment, ventral aspect.

lateral angles, but hardly quite as much so as in *vernalis*. The next to last thoracic segment does not have its posterior margins produced laterally, being thus unlike that of *vernalis*. The genital segment, as long as the 3 following segments taken together and expanded prominently at the sides near the anterior margin, narrows rapidly behind these prominences to the line of partial fusion of first and second segments, while that part of the genital segment representing the second abdominal segment has parallel lateral margins. Beyond the genital segment there is a very slight taper to the base of the furca. The anal plate extends far back (fig. 5 *d*), at least halfway and sometimes fully to the base of the furca, sometimes a little beyond, this being one point of distinction as compared with *vernalis*.

The posterior margins of the abdominal segments, except the last, are conspicuously dentate and fine spinules are observable on the posterior margin of the last abdominal segment. Pronounced dentation of abdominal segments is characteristic of juvenile stages of *vernalis* and therefore this offers a point of relationship. The furcal rami (fig. 5 *j*) nearly parallel, of approximately uniform width throughout and $\frac{1}{4}$ to $\frac{1}{3}$ as wide as long, bear series of fine hairs on the interior margins (their strength exaggerated in the drawing); a slight notch, often armed with a spinelet, is found on the outer margin about $\frac{1}{3}$ way from back. The "ciliation" of the rami may be thought of as a feature of relationship to *viridis* Jurine. Of the two well developed terminal setae the more mesial is about $\frac{1}{3}$ longer than the other and rather longer than the abdomen, or from 0.4 to 0.6 the length of the body.

The first antennae (fig. 5 *a*), about as long as the first segment of the body, are 12-jointed, the first 6 segments and the last 3 being as in *vernalis*, while the relatively long 8th and 9th segments correspond, respectively, to the 8th–11th and the 12th–14th of that species; they show no trace of subdivision. The second antennae and mouth parts seem to be identical with those of *vernalis*.

Both rami of P_{1-4} are 3-jointed, with spine formula for the distal segments of exopodites 2–3–3–3, in all specimens that we have examined. The full spinous armament is attained in the 4th copepodid stage; the number of segments in the exopodites of the swimming feet increases from 2 to 3 at the molt from the 5th copepodid to the adult, but no additional spines are gained. The distal segment of the exopod of P_4 has width and length in about the proportion of 5:8 (fig. 5 *g*). The proportions of the distal segment of the endopod of this limb (fig. 5 *h*, *i*) have been regarded as of significant diagnostic value; in this copepod

we find them quite variable, the greatest width being usually from 54 to 66 per cent (in one case 51 per cent) of the length, but if the appendage is viewed flat, the length is never "more than twice the width," as is commonly the case with *vernalis* and as is reported for *venustus* by Kiefer (1929). The two terminal spines are always clearly unequal, the inner spine being the smaller, its length $\frac{3}{5}$ to $\frac{3}{4}$ that of the outer. The fifth foot (fig. 5 *b*) is practically identical with that of *vernalis* (fig. 1 *c*), the basal segment twice as wide as the distal, or a little more, the distal segment narrow, and relatively long, with the spine on the mesial side inserted near but not at the tip, a chief point of distinction between *vernalis* Fischer and *viridis* Jurine (with spine far from the tip).

The form of the seminal receptacle is variable but characteristic (fig. 5 *e*, *f*). The anterior portion is much like that of *vernalis*, its anterior margin convex, sometimes flattened or even slightly concave near the middle; posteriorly the organ is more complex than that of *vernalis*, there being, in addition to the usual transverse portion, a deeper (more dorsal) pocket that extends posteriorly in the middle region; the depth and complexity of this pocket is quite variable, but it is always present in some form; the relation of this pocket to the transverse portion and to the anterior portion is not altogether clear. The kidney-shaped spermatophores resemble those of *vernalis*. Egg-sacs are very variable; sometimes there are only 3 or 4 large eggs, loosely attached to each other; sometimes there are about a dozen large eggs in a good sac, and again there may be large well-formed sacs having some 50 eggs in each.

Males are so much like those of *vernalis* that we have not attempted to distinguish those collected in nature. When we have compared males reared from eggs of females of this species with those reared from eggs of examples of *vernalis*, we find the anal operculum, as in females, extending much farther back than in males of *vernalis*, the ciliation of the furca faint or wanting, and the dentations of the abdominal segments inconspicuous. The furcal setae are distinctly longer than those of females, but the longest are not quite $\frac{2}{3}$ the length of body.

The lengths of females collected in nature were 1.17–1.56 mm., of reared females 1.01–1.13 mm. Reared males were about 0.9 mm. in length. The length of the furca relative to length of body exclusive of caudal setae varied from 0.085 to 0.10, and the width of the furca relative to its length from 0.23 to 0.30; roughly speaking, the furca is about one-tenth as long as the body and each of its branches about one-fourth as wide as long, or a little wider.

This copepod has been found in the vicinity of Chapel Hill, N. C., in two marshy meadows, in one of which we have never failed to find it in winter, and in a spring run in the forest where there was a little water among an abundance of leaves. In each place it was associated with *C. vernalis*.

Type in United States National Museum, No. 69103.

Systematic position of *venustoides*

The copepod resembles *vernalis* Fischer in the structure of the 5th foot as well as in the general form of the body. It departs from that species, not only in the number of segments in the first antenna, but also, and we regard this as of more importance, in the form of the next to last thoracic segment which lacks the postero-lateral projections prominent in *vernalis* and seemingly an invariable characteristic of that species. It differs from *vernalis* also in the form of the seminal receptacle, in the possession of conspicuous dentations on the posterior margins of the abdominal segments, wanting or inconspicuous in adult *vernalis* (although quite prominent in juvenile stages), and, generally, in the decided inequality of the terminal spines of the endopodite of P_4. There is much diversity of form and armature in the terminal segment of that member in both species, but, withal, the relative equality of the terminal spines seems remarkably constant in *vernalis* and the conspicuous inequality as constant in this copepod.

Norman and Scott (1906) described a new copepod, *venustus*, strongly resembling *vernalis*, but having only 12 segments in the first antenna. The original description is not complete, and some of the differences between our copepod and theirs seem of minor importance. In ours the distal segment of P_5 is much longer and narrower than shown and described for *venustoides*, and more irregular in outline. The authors show no spinule or notch on the outer margins of furcal rami, but the notch is often quite inconspicuous in our copepods; they make no reference to abdominal dentations, but these are reported by Kiefer (1929) and Dr. Gurney informs me by letter that the dentations are a chief point of distinction of *venustus*. Kiefer gives the spine formula as 3–4–4–4, instead of 2–3–3–3 as in ours, but this is probably of slight taxonomic significance. Figure 1 of Norman and Scott indicates that the anal plate extends relatively far posteriorly and that the rostrum is quite prominent, features in which our copepod conforms with theirs.

Through the courtesy of Dr. Gurney, I have had the opportunity to examine an adult female and some immature examples of *venustus* from Great Britain. The adult female conforms with our copepod and differs

from *vernalis* in the conspicuousness of the dentations of the abdominal segments, the ciliation of the mesial margins of the furca (possibly in the form of the seminal receptacle—this is not clear), in the proportions of the terminal segment of P_4 endopod (W/L = 0.52), and in the posteriorly placed anal plate. It differs from *venustoides* in the spine formula (not important) and more notably in the form of the next to last thoracic dorsum, which is like that of *vernalis*, and in the relative lengths of the terminal spines of P_4 endopod, which are as in *vernalis*. Furthermore, the distal segment of the 5th foot is much wider than in our copepod; fig. 5 *c*, drawn from the example in hand conforms well with the original figure and description of Norman and Scott; the length of the segment is not over 1½ times the width, whereas in ours it is twice the width. The longest furcal seta, while not nearly equalling the length of the body, as noted by Kiefer, is much longer than in ours: its length is 71 per cent of that of the body, while in ours it is 40 per cent to 60 per cent.

Venustus is evidently intermediate between *vernalis* and *venustoides*. We need not altogether dismiss the possibility that both *venustus* and *venustoides* are varieties of *vernalis*, or that *venustoides* is a subspecies of *venustus*, but, measured by the best existing taxonomic criteria, each is clearly a distinct species. Furthermore, we have found that *venustoides* breeds true and that it is very different from *vernalis* physiologically.

Physiological peculiarities of the species

This copepod at Chapel Hill is found regularly associated with those we have identified as *vernalis*, but the two species react very differently to conditions of temperature. *Vernalis* develops very rapidly at room temperatures (23°C., cir.), completing a life cycle, from egg to egg-bearing female in 7 to 10 days. At about 7°C. the female attained maturity in 65 days. What is evidently the same species reared at Paris completed a life cycle in 11 days at 22°, and, at an average temperature of 9.5° attained maturity in 30–50 days, a close enough correspondence with the copepods of the same species at Chapel Hill. *Venustoides*, reared at Chapel Hill simultaneously with *vernalis* and in identical culture media, took 3–5 times as long to attain the 4th copepodid stage, and, in all our experiments, failed to complete development at room temperature, although the 4th copepodid stage lived even better at relatively high temperatures than did those of *vernalis*. At about 7°C., and possibly at somewhat higher temperatures, it completes development by molts at long intervals. We have not had viable eggs

or viable early nauplii except at low temperatures. *Venustoides* is, then, distinguished from *vernalis* in that, while the latter is broadly eurythermal at all stages, the former is eurythermal only at certain stages, being narrowly stenothermal in respect of development through the last two copepodid stages. More complete data regarding these experiments are given in another place (Coker, 1933). From its rate of development and response to temperature, *venustoides* must be supposed to be monocyclic, or at most dicyclic, as contrasted with highly polycyclic (or acyclic) *vernalis*.

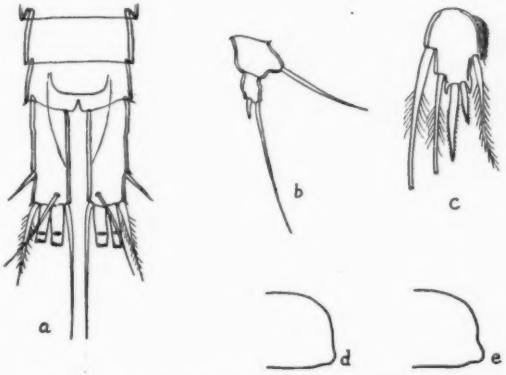

FIG. 6. *Cyclops exilis* n. sp. (a) furca and posterior segments of abdomen; (b) fifth foot of female; (c) terminal of endopod of fourth foot; (d) lateral margin of next to last thoracic dorsum; (e) same, an extreme case of prominence.

Cyclops exilis, n. sp.

In spring runs, both at Chapel Hill, N. C., and at the Allegany School of Natural History in Cattaraugus County, N. Y., there occur in considerable numbers egg-bearing copepods, very similar to *venustoides* in form of body and antenna, but much smaller and generally with 11 segments (occasionally with 12) in the antenna. The missing segment is the short third segment characteristic of all copepods of the group having 12 or 17 segments. In one example the second joint is incomplete, so that, while 11 segments may be counted on the anterior margin of the antenna, only 10 may be counted on the posterior margin. The examples from Chapel Hill, which have been studied more closely, have most of the characteristics of *venustoides*, as to form of seminal

receptacle (although this is very difficult to make out clearly), fifth foot, margins of thoracic segments, anal operculum, relative lengths of caudal setae, etc. They differ *obviously* from the species chiefly in characters that might be thought to be associated with dwarfing, and which we do not, therefore, like to regard as having either specific or subspecific significance. The segments of the antennae, especially the distal six, and all segments of the swimming feet are short and relatively broad; the parallel furcal rami are likewise short and broad: length of furca is 0.07–0.08 length of body, and ratio of width to length of each ramus is 0.38–0.42. The mesialmost apical furcal seta is very slender

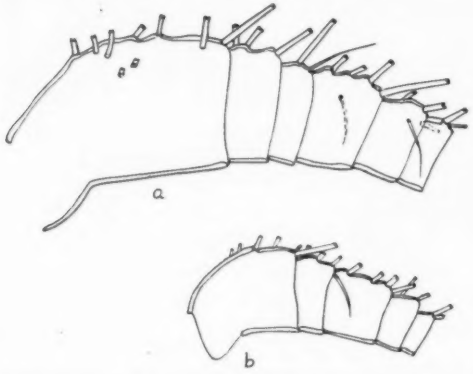

FIG. 7. Proximal segments of antennae of—(a) *Cyclops vernalis* Fischer, and (b) *Cyclops exilis*.

and long, nearly twice as long as the outermost, which is about two-thirds as long as the furca (fig. 6 *b*); of the two well developed apical setae, the inner is nearly twice as long as the outer, which is about as long as the abdomen. Relative lengths of the apical setae are about as in *venustoides*, but the setae are much longer relative to length of body. The postero-lateral angles of the next to last thoracic segment are not distinctly turned outward, as in *vernalis*, nor so well-rounded as in *venustoides*, but there is generally a more or less definite suggestion of prominence at this angle (fig. 6 c). Interior margins of furcal rami, unlike those of *venustoides*, are bare. Posterior margins of abdominal segments are scarcely if at all dentate.

Experience in rearing *Cyclops vernalis* under different conditions of temperature (Coker, 1934 and 1934a) would readily lead us to suspect

that most of the differences so far mentioned might be attributable to conditions prevailing during development, that the form is an ecological variant; but there is a more decisive point of structural distinction. The two apical spines of the endopod of P_4 are just as unequal as in *venustoides*, but the relations are exactly reversed: the *mesial* spine is invariably the longer. It is conceivable that this difference is attributable primarily to environmental rather than to genetic conditions and we had hoped to submit this question to experimental test. Unfortunately, although females form or carry egg-sacs freely in laboratory cultures, we have not so far been able to rear the copepod at low, intermediate or high temperatures; this copepod has not responded favorably to cultural conditions that have given results with the other species.

There seems to be no alternative but to give the form a name, whether as species or as subspecies seems relatively unimportant. We have collected the copepod year after year and the characters are quite consistent; we have found no examples making the transition to *venustoides*, the form that it most resembles. The larger copepod, with the 12-jointed antenna and ciliated furca, always has the inner apical spine of P_4 endopod decidedly shorter; the smaller form with 10–12-jointed antenna (almost always 11-jointed), with dwarfed appendages and dwarfed and non-ciliated furcal rami, always has the inner apical spine decidedly longer. We apply the name *exilis*, meaning dwarfish.

Length: Females, Chapel Hill—0.78–0.85; New York—0.88.

Mrazek's *michaelseni* seems to have the lateral furcal spine more proximally placed, the furcal rami divergent and a different form of seminal receptacle. Chappuis's *kieferi* has the mesialmost apical furcal seta short and spinelike, instead of notably longer and slender. Chappuis's *reductus* has the rami of the first three swimming feet 2-jointed, instead of 3-jointed. (See Kiefer, 1929). Only further breeding experiments could determine the exact status of the several forms here considered, whether they are all species or only ecological variants of one species.

Type in United States National Museum, no. 69104.

SUMMARY

Copepods originally identified as *Cyclops americanus* Marsh from Chapel Hill, N. C., and others identified as *C. robustus* Sars from the vicinity of Paris, France, have been reared for several successive generations in each case. Comparisons have been made with wild copepods

from the regions of Chapel Hill and Paris. The diversity of forms obtained in breeding compels the identification of both sets of copepods with *C. vernalis* Fischer. *Cyclops parcus* Herrick and *C. brevispinosus* Herrick are also assigned to that species. In part this is confirmatory of the views of some European investigators, although it is contrary to existing American practice.

There is described from Chapel Hill a new species, *C. venustoides*, intermediate perhaps, between *vernalis* and *viridis* Fischer, but differing markedly from both. It is nearly related to *C. venustus* Norman and Scott, which appears to be intermediate between *vernalis* and *venustoides*. Another form, with (generally) 11-segmented antenna and otherwise consistently distinct from *venustoides* (with 12-segmented antenna), is provisionally designated as a new species, *exilis*.

Vernalis breeds in laboratory cultures and matures with great rapidity at room temperature, the cycle from egg to egg being completed in 7–10 days; a period about 7 times as long is required at a temperature of about 7°C.

The period of development for *venustoides* seems to be 3–5 times as long as for *vernalis* at corresponding temperatures. Furthermore, while *vernalis* is broadly eurythermal at all stages of development and *venustoides* equally so at intermediate stages, the eggs and early nauplii of the latter species lived only at low temperatures in our experiments and arrest of development in the 4th copepodid stage (10th larval stage or second from last) was indefinite except at low temperatures, as we had previously reported before the species could be described and named. Although generally broadly tolerant as to survival at all ordinary temperatures, *venustoides* displays a peculiar sort of stenothermy as to development through certain stages.

The form described as *exilis* has not yet responded favorably to any attempts to breed it in the laboratory.

The group of which *Cyclops vernalis* Fischer is the type remains a very complex and puzzling one. A question that originally prompted our experiments was this: to what extent are the many slightly different forms described as species, subspecies or varieties attributable to extraordinary plasticity within a species or a tendency to explosiveness, genetically speaking? The experiments and observations, for which only the taxonomic aspect is reported here, have thrown some light on the subject, resulting in some simplification and tending to the elimination of names; but it has also added new names, temporarily, at least.

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A ROT OF PEAR CAUSED BY THE RED BREAD-MOLD FUNGUS

By Minerva Waynick

Two Text-figures

The red bread-mold fungus, *Neurospora sitophila* Shear and Dodge, has long been known to infest bakeries in France, Italy, Germany, and the United States. As a result of such infestations this mold has been extensively studied. As additional habitats, it has been reported to occur on such substrata as on burned tree trunks and on various fruits. Apparently, however, no studies have been made of it as a fruit-rotting organism. It seemed desirable, therefore, when this organism, in its conidial stage, appeared as a soft rot of pear fruits obtained on the market in Durham, North Carolina, to make a study of its parasitism and the nature of the decay which it incites.

Pure cultures were first obtained from the growth resultant from transferring to potato agar, tissue from decaying pears. Subsequent isolations of the same organism were made from other decaying pears and from the surface of pots of sand which had been autoclaved and on which the fungus appeared fortuitously. Identification of the causal organism as "Sex B" of his monilioid, pigmented form of *Neurospora sitophila*, was made by Dr. B. O. Dodge.

In order to secure further evidence of the ability of this mold to rot fruits, pears and apples were disinfected with a solution of calcium hypochlorite and placed in moist chambers. The inoculum, consisting of hyphae and conidia, was introduced through incisions made with a sterilized scalpel. Some of the inoculated fruits were stored at a temperature of 20°C. and others at 35°C. Brown, decayed areas surrounded the points of inoculation on the pears after two or three days. For some unknown reason, no lesions appeared on the apples. Within the next few days a loose, orange-colored mycelium, bearing masses of conidia covered the surface of the lesions (Fig. 1). Reisolations were very readily accomplished.

Diseased tissues, when teased apart in a drop of water or when subjected to pressure under a superimposed cover glass showed that the

pulp cells tend to separate intact and that the mycelium is intercellular. These observations were verified by examination of paraffin sections cut at a thickness of 40 microns. Normal pulp cells of pear fruits consist of large, succulent parenchyma cells interspersed between groups of stone cells (Fig. 2, A). In sections of diseased tissue the hyphae may

FIG. 1. NEUROSPORA SITOPHILA ON DECAYING PEARS

be observed to course between the parenchyma cells and to be so abundant as to more or less completely outline the cells in the invaded areas (Fig. 2, C). No evidence of intracellular invasion was noted. The fungus causes a dissolution of the middle lamella slightly in advance of the invading hyphae, which results in the separation of cells (Fig. 2, B).

Following this, the cell walls disintegrate and the cell contents are utilized by the parasite. These contents appear to consist mainly of reducing sugars, as indicated with Benedict's test, and of organic acids. Starch is absent.

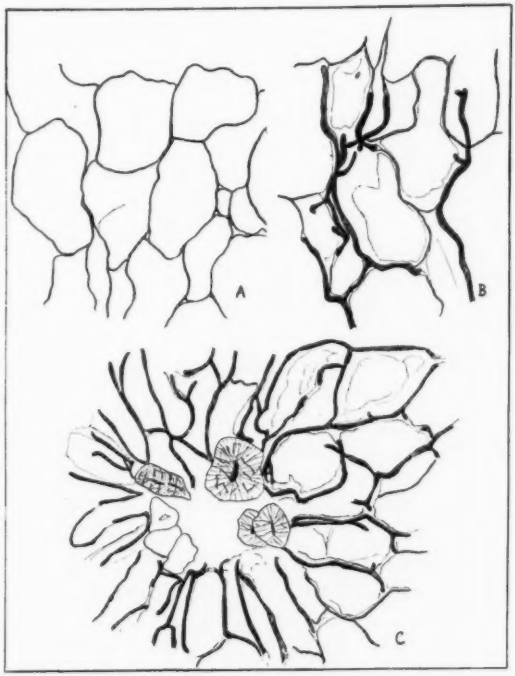

FIG. 2. SECTIONS OF TISSUE FROM PEAR FRUIT

(1) Normal uninfected cells. (2) Intercellular mycelium among cells whose cytoplasmic layer has shrunken, and whose walls are being dissolved. (3) Cells outlined by intercellular mycelium.

A study of the activity of *N. sitophila* in culture was made to confirm the microscopic findings. When soluble starch in starch agar constituted the sole nutrient, the fungus made a sparse growth and there was no evidence, as shown with iodine, that appreciable quantities of starch had been utilized in cultures one week old. Potato agar, however, supported a luxuriant growth. Transfers of hyphae to this medium

resulted in growth that bore new crops of conidia within 4 or more hours. When the initial reaction was pH 7.4 to 7.6 in tube cultures on potato agar and brom thymol blue was used as an indicator, a broad yellow zone formed at the surface of the agar within 24 hours, indicating production of acid. As growth continued this yellow zone extended to the bottom of the tube.

Mycelial growth is not confined to the surface of the agar but is almost equally luxuriant within the agar to a depth of 15 to 20 cm. When melted cooled tubes of slightly alkaline potato agar are inoculated with conidia and are then agitated to distribute the inoculum throughout the tube, mycelium develops throughout the entire length of the tube, and the reaction changes to acid within two or three days. This fungus seems to grow well as a facultative anaerobe—a fact which has been previously noted and which is worthy of critical study.

Acid formation occurs both in 1 per cent dextrose and in 1 per cent sucrose agar when the initial reaction is alkaline, showing that both sugars can serve as food for this mold. When the medium containing either of these sugars is slightly acid at time of inoculation, a blue layer forms just at the surface of the medium and persists for a week or more.

Pectin, one of the pectic constituents in pears, does not appear to be utilized in quantity. This was shown by failure to secure marked increase in acidity resultant from growth on alkaline pectin agar. Pectin agar was prepared according to a method used by Wolf[1] in which "Certo" was purified by repeated precipitation with alcohol. It is very apparent, however, as shown by examination of tissues, that the primary membranes of the pulp cells, which are constituted of calcium pectate, serve as a food substance. Presumably protopectins and certain of their cleavage products can also be utilized.

SUMMARY

Neurospora sitophila causes a soft rot of pears on the market. Decay is accomplished by dissolution of the primary membrane, after which the cell contents are utilized by the parasite.

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[1] Wolf, F. A. Studies on the physiology of plant pathogenic bacteria. VI. Pectic fermentation in culture media containing pectin. Phytopath. **13**: 381–385. 1923.

NOTES ON THE EGG-LAYING AND NESTING HABITS OF CERTAIN SPECIES OF NORTH CAROLINA MYRIAPODS, AND VARIOUS PHASES OF THEIR LIFE HISTORIES

By William S. Cornwell

The data for the following notes on the egg-laying and nesting habits and various phases of the life histories of certain species of Myriapods were taken at various times during the months of June and July, 1933, by various collectors from the Zoology Department of Duke University. Most of the evidence was obtained from the Duke Forest although some of it came from the vicinity of Durham, N. C., outside the Duke Forest and from elsewhere in the state of North Carolina.

JULIDAE

Spirabolus marginatus (Say). Collected by Dr. A. S. Pearse, June 3, on Bent Creek near Asheville, North Carolina. Two nests were found. In one, located under a log, a big Spirabolus was lying on top of 480 separate cocoon pellets, of which each of those examined proved to contain an egg. In the other nest, also under a log, 90 cocoon pellets were discovered. The nests may be described as holes in the ground with no upper covering of earth, the body of the female and the logs under which the nests occurred serving that purpose.

The cocoon pellets, which were dark brown in color, measured 5 mm. by 4 mm. They seemed to be composed of organic soil material or possibly excrement. The spherical white eggs were very small in comparison with the pellets, measuring only 1 mm. in diameter.

Additional evidence of the egg-laying activities of *Spirabolus marginatus* was found in a swampy area adjacent to the Eno River, near Durham. Here the nests of Spirabolus seemed to be located in the logs themselves. Several of these animals were found in cavities underneath the bark of greatly decomposed logs. In numerous cases many pellets were found which apparently contained no eggs and seemed to be only excrement. Other pellets of similar nature did contain eggs. These observations were made in late July.

SCOLOPENDRIDAE

Scolopendra viridis Say. At different times during the months of June and July, five females of this species with eggs were found in the Duke Forest or in the vicinity of Durham. On June 27, a female with 40 eggs was found by Mr. N. E. Rice in a cavity in the cambium layer of a somewhat decomposed oak log. The nest may be described as open. Apparently the female had made use of an insect passage way in the wood, doing nothing itself by way of construction. The eggs were attached in a mass to the abdomen of the extended female. A gelatinous matrix of very thin consistency seemed to hold the eggs together and in turn attach the mass to the female's abdomen. These eggs were a little larger than those described for *Spirabolus marginatus* and were ovate rather than spherical; length, 2 mm.; width, 1.5 mm.; color, light yellow.

A second female with 46 eggs was collected on June 29. Again the nest, in a partly decomposed and very large oak log, was located on the upper side beneath the cambium bark. The position of the eggs under the abdomen was the same as previously described but the female was curled up. The temperature of the log both under the bark, which was somewhat shaded, and in the cavity in which the nest was located was 27°C.

The other three females with eggs were taken by Mr. R. G. Taylor. The first, July 3, was a small female with 26 eggs. It was found underneath a quite decomposed loblolly pine log. Again the eggs were attached to the abdomen. The other two females, both of which were of small size as compared to the first two mentioned had 19 eggs and 30 eggs, respectively, attached in masses to the abdomens of the females. These females, with eggs, were also taken from under the bark of loblolly pine logs. The size and color of the eggs were the same as previously mentioned.

On July 27, 1933, Mr. R. G. Taylor found a female with 19 eggs attached to the abdomen, and 26 young clasped on her ventral side. The female was observed in a small cavity underneath the outside bark of a loblolly pine log. The young were white in color and about 6 mm. long. They appeared to have the complete number of legs, which is 21 pairs. The eggs were even somewhat more oval in outline than those previously described but were of about the same size.

CRYPTOPIDAE

Cryptops hyalina Say. On July 5, a female with 9 eggs was discovered beneath a not greatly decomposed loblolly pine log. The nest was on the outside of the bark between the log and the ground. In it the temperature was 25°C. The eggs were in a mass next to the bark and the female was curled up on top of them. Minute in size, and oval in shape, these eggs average about 0.75 mm. in length and about 0.5 mm. in width. The color is light brown.

On June 15, two females with young were collected in the Duke Forest. The first found was curled around 9 young on the underside of a loblolly pine log beneath the outside bark. The second was found beneath the bark on the side of an oak log. This female was curled around 10 young.

On July 8, while making an investigation for the occurrence of Myriapoda in the soil, a nest of a cryptopid was found in the forest floor just beneath the humus layer of a loblolly pine standing in the Duke Forest. There were 9 young present all curled up together, but the female was absent, perhaps driven away by the digging operations. The texture of the soil in which the young were discovered was silt loam. The litter above them was composed exclusively of pine needles. The nest can be described as nothing more than a cavity in the soil, showing no apparent activity on the part of the female towards nest construction. While absence of the female prevented accurate identification of the species, the young so far as the observations could be carried were identical with those described for *Cryptops hyalina*.

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SPIROCHETES IN THE CAT WITH SPECIAL REFERENCE TO THOSE OF THE ALIMENTARY TRACT[1]

By Eloise E. Greene

Plates 21 and 22

INTRODUCTION

The purpose of this paper is to review the types of spirochetes in normal cats, as they are found in the alimentary tract, especially in the mouth, caecum, and large intestine, through study of the fresh material under the dark-field and through the study of fixed stained specimens. A survey of the principal organs including the heart, lungs, liver, spleen, and kidney, is included. The filtration and cultivation of the spirochetes and their implantation in young cats are also dealt with.

Summarizing the historical data, it will be noted that most of the work done on the spirochetes of the cat has involved those of the stomach. Salomon's (16) work deals with the three gastric types. Though Escherich (5) mentioned the fact that spirochetes were found in the caecum and large intestine, he described only a coarse corkscrew form. MacFie (10) noted a type found in the large intestine and rectum which was very loosely coiled. Sanarelli (17) observed that spirochetes lived especially in the large intestine. DeMello and Fialho (4) noted and illustrated only one type similar to the *Spirochaeta eurygyrata*, the human intestinal spirochete. Nowhere in the literature is there a complete survey of the spirochetes of the cat. Only the spiral forms found in the stomach and one type found in the large intestine are described. No reference to the types of spirochetes in the mouth was found.

EXPERIMENTAL WORK

The study of spirochetes in the cat was divided into the three following divisions: 1. Study of the living material under the dark-field, and observation and measurement of fixed stained specimens. 2. Filtra-

[1] From the Department of Bacteriology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore. The writer wishes to acknowledge the assistance given by Dr. W. W. Ford and Dr. L. B. Lange in this investigation.

tion and cultivation experiments. 3. Implantation experiments in young cats *per os* and *per rectum*. All work was done on apparently healthy cats secured from various sources. Most of the animals were killed immediately, but a few were kept from 2 days to a week in the laboratory until needed. The animals were not fed for approximately 15 hours preceding the examination.

1. Study of living material under the dark-field and observation and measurement of fixed stained specimens

The animals were killed in the laboratory either by ether or gas and the entire alimentary tract removed. The oesophagus, stomach, caecum, and large intestine were each removed with uncontaminated instruments and placed in separate Petri dishes. The small intestine was roughly divided into duodenum, jejunum, and ileum and the three parts placed in separate Petri dishes. The oesophagus, stomach, and intestines were each opened with a pair of sterile scissors and the contents washed out with physiological salt solution. Studies were made under the dark-field of the contents, and of the mucosal scrapings emulsified in Ringer's solution.

A. Living material

Under the dark-field four distinct types of spirochetes, the spironeme, treponeme, fusi-spiral, and leptospira, were found in the living material.

Adult cats. Forty-eight out of fifty adult cats contain spirochetes in the mouth or gastro-intestinal tracts or both. Spironemes, treponemes, fusi-spirals, and in a few cases leptospiras were observed in the mouth, caecum, and large intestine, the spironemes and treponemes usually in large numbers. Fusi-spirals were scarce in the mouth, but abundant in the caecum and large intestine, especially in the caecal scrapings. Leptospiras were never numerous.

a. Mouth: The organisms differed in size and proportions but four common types stood out. The most common was a spironeme (No. 1), approximately 10μ long and 0.5μ thick when stained with the Kliewe and Kliewe-Harris stains. It is usually extremely flexible, the spirals in many cases undergoing frequent change. Some individuals of this type were quite sluggish, sometimes remaining motionless for short periods before resuming their characteristic movement. There are 3 to 6 rather shallow, irregular curves and the ends are sharply tapered (Figs. 1 and 2). In quiescent forms the spirals were more regular. This spirochete was classified in the present summary as a spironeme,

because of its great flexibility and the frequent change in the form of the spirals, relatively shallow and few in number, together with a greater bending and lashing than is seen in the typical treponeme. Though most of the spironemes were from 10μ to 15μ long, many were 20μ to 30μ and some even longer (Figs. 2 and 3). These were evidently in the stage preceding division as shown in Fig. 2. Their thickness was usually 0.5μ.

A second spironeme (No. 2) often observed in the mouth appeared under the dark-field as a long thick organism with a definite double contour and wide, flat curves. It is from 10μ to 30μ long and 0.75μ to 1μ thick (Fig. 2). A similar organism, obtained from the human mouth, was described by Noguchi (15).

A third spironeme (No. 3) is that shown in Fig 4b. This organism was present in the mouth of eleven cats, in the caecal scrapings of two cats, and in the colonic scrapings of one cat. This organism is approximately 20μ to 25μ in length and has small, regular, primary spirals and usually two secondary curves of 5 to 6 primary spirals each. The latter remain practically fixed, but there appears to be a continuous change in the primary spirals. The rotation of the body with the resulting progression of the light reflex gives the impression of a wave-like motion along the length of the organism. When stained, both the secondary and primary spirals usually become irregular (Figs. 3 and 4). The ends are pointed. It resembles the *Spirochaeta pseudorecurrentis* of Zuelzer (21), a water spirochete probably with a number of subspecies (Fig. 4a).

A fourth spironeme (No. 4) was noted in fresh material from the mouths of 11 cats. It also occurred in the ileal contents of a kitten about 10 weeks old, and in the contents and scrapings of the caecum of an adult cat, where it was clearly observed under the dark-field, and in fixed, stained specimens among the more common spironemes mentioned above. It is a typical spironeme approximately 10μ long and 0.3μ to 0.5μ thick, with 3 to 6 spirals which are fairly constant when the organism is in motion and maintain their form in fixed preparations (Fig. 5). The spiral depth and amplitude is 2μ. The ends are pointed but no terminal filaments were seen. A small projection appears at the apex of each rounded spiral, clearly visible under the dark-field and in the films stained by the Kliewe-Harris method. The regularity of these projections and their constancy despite rotation of the spirochete suggest that they may represent a thin lateral extension of the body, or a regular series of protrusions too small to distinguish individually, arising

from the convex surface of the spirals along the length of the organism. Such a continuous or intermittent ridge might account for the microscopic picture. One would expect such a ridge to be seen clearly where it would protrude from the apices of the spirals into a vacant background, but to be seen less clearly if at all, between the apices where, as viewed by the observer, it would lie above or below the body of the spirochete. While the projections from the apices of the spirals appear in stained specimens as they do under dark-field illumination, it has not proved possible up to the present to demonstrate a hypothetical ridge along the body between the spiral apices by any of the staining methods used in this work. A thorough search of the available literature has not disclosed a record of this morphological type. The name *Spironema langei* n. sp., is suggested for this spirochete.

The small lateral protrusions of this spironeme are different from the "knospen" or buds of Meirowsky (12), Fig. 6, in that they appear regularly at the apex of each spiral turn and are delicate and pointed while the "knospen" occur irregularly on the end and laterally near the middle of the spirochetes, and are rounded. Meirowsky gives a good survey on these "knospen." They were considered by some to be a propagation phenomenon and by others a degeneration process. In Fig. 7 an example of the "knospen" is shown on a spironeme from the mouth of the cat seen in the course of this work, which is similar to No. 53 of Fig. 6, Meirowsky's sketches illustrating the "knospen."

As contrasted with the spironemes which were definitely of distinct morphological types, the treponeme group was made up of organisms of various sizes which seemed to grade into one another. The treponemes of the mouth varied in length from 5μ to 15μ, exceptionally 15μ to 20μ. Most of them averaged 5μ to 10μ, the greatest number being 5μ long. A few long individuals were evidently about to divide. The more numerous type (A) was 5μ long and 0.25μ thick, and the less frequent type (B) 10μ long and 0.25μ thick, that is, twice the length but the same diameter (Fig. 8). These measurements and the fact that incurvation forms (Fig. 9) were common among the longer individuals suggest that they constitute one species undergoing rapid transverse divisions. A few organisms were somewhat thicker (Fig. 10) and appeared to be a distinct species. The observations did not permit further conclusions as to the number of species represented by these forms. All the principal types of treponemes were found in the mouth.

Leptospiras were found in a few cases in mouth material. They were approximately 10μ long, very slender, and had extremely flexible hooked

ends. They were noted only occasionally, were few in number, very active, and not always easily detected in a field containing numerous spironemes and treponemes.

b. Esophagus: Though spirochetes of various types were found in great numbers in the mouth, none were observed in the esophagus, although in every animal scrapings emulsified in saline were carefully examined.

c. Stomach: In the gastric contents no spiral organisms were seen, but in the mucosa of 11 cats there was a rigid, coarse organism with closely-set spirals of uniform depth and with abruptly pointed ends. It was not encountered at any other site. In an emulsion of the pyloric mucosa of cat No. 4 a very delicate treponeme resembling *Treponema pallidum* was found. This treponeme appeared more delicate than treponemes A and B mentioned above, both under the dark-field and in fixed stained specimens.

d. Small intestine: The spirochetal flora of the small intestine was scanty. A few spironemes were seen in the duodenal contents of cat 46, the jejunal contents of cats 3, 46, and in the ileal contents of cats 5, 25, 46. Short treponemes, approximately 5μ long, with a spiral depth of 0.6μ to 1μ were found in the ileal contents of cats 13, 44. No spirochetes were observed in the mucosal scrapings of the small intestine.

e. Caecum and large intestine: Treponemes, spironemes, fusi-spirals, and leptospiras were found in the contents and scrapings of the caecum and large intestine. The first three types were found in larger numbers in the mucosal scrapings than in the contents. The spironemes and fusi-spirals were more numerous in the caecal scrapings and the short treponemes were more numerous in the colic scrapings. The majority of the spironemes and treponemes were morphologically similar to the two common forms in the mouth (Spironeme No. 1 and Treponeme A). The longer dividing forms of spironemes and treponemes were not as usual in the caecum and large intestine as in the mouth. Only one spironeme dividing was seen in the caecal scrapings of cat 29. The fusi-spiral was observed in the contents and scrapings of the caecum and large intestine, in greatest numbers in the caecal scrapings. In fixed stained specimens it was approximately 10μ long and 0.5μ thick in the middle, tapering gradually toward the sharply pointed ends (Fig. 11), and the spirals were usually irregular. Sometimes they were regular and shallow, one or two in number, with a depth of 2μ and an amplitude of 4μ. The movement of this organism is very characteristic. While rotating and advancing it exhibits the typical spiral form

as the reversed motion commences. Sometimes it assumes a circular form with loss of spirals. In our material we also observed, occasionally, the posterior end to become fixed and the anterior end to lash backward forming a circle (Fig. 11). After brief arrest the anterior end would spring forward and the spirals reform as the organism shot forward in the original direction.

As in the mouth, leptospiras were noted only a few times, being found in the mucosal scrapings of the caecum and large intestine of 5 cats. They were approximately 10μ long, very slender with very active hooked ends. They were seen only in small numbers under the dark-field and in fewer numbers in stained preparations.

Summary of the findings in adult cats according to types of organisms: Spironemes and treponemes are the most common types throughout. The commonest spironemes (No. 1) are approximately 10μ long and 0.5μ thick with 3 to 6 irregular spirals, the commonest treponemes (A) are approximately 5μ long, 0.25μ to 0.3μ thick, with 4 to 8 regular close-set spirals. The treponemes and spironemes from the mouth, caecum, and large intestine were indistinguishable morphologically either under the dark-field or in fixed stained specimens. Fusi-spirals, leptospiras, and a coarse rigid spiral organism (stomach) are less common.

Summary of the findings in adult cats according to distribution in the animal body: In the mouth, spironemes, treponemes, fusi-spirals, and leptospiras were found. The most common forms were the spironemes (No. 1) and the treponemes (Type A). In the esophagus no spirochetes were noted. In the mucosal scrapings of the stomach, a few spiral organisms were observed. They were rigid, coarse, with closely-set spirals of uniform depth, and abruptly pointed ends. This type was not seen in any other part of the alimentary tract. In the pyloric wall of one cat a few treponemes (similar to *Treponema pallidum* and more delicate than Types A and B) were demonstrated. Rarely the contents of the small intestine showed a few spironemes and a very few treponemes. Fusi-spirals, spironemes, and treponemes were found in large numbers in both contents and scrapings of the caecum and large intestine. In none of the 84 cats examined were spirochetes found in the lungs, liver, spleen, heart, or kidney.

Kittens. The kittens studied ranged in age from mature embryos to approximately 90 days. In the 22 apparently normal kittens examined no spirochetes were found in the alimentary tract of those younger than 60 to 90 days. The type most often noted in older kittens was the spironeme, which was especially numerous in the mucosal scrapings of

the caecum of two kittens. Spironemes were also found in the large intestine, more numerous in the mucosal scrapings than in the intestinal contents. Treponemes were found in a few cases in the scrapings of the large intestine, usually in moderate numbers but in one case they were very abundant. Treponemes were not observed in the contents of the large intestine. In none of the 22 apparently normal kittens were spirochetes noted in the liver, lungs, spleen, heart, or kidney.

B. Stained material

In staining the organisms, it was found relatively easy to stain both the spirochetes from the mouth and those from the caecum and large intestine by many different methods including Giemsa, Fontana-Tribondeau (20), Kliewe (9), Kliewe-Harris (6), Romanowsky (8), Medalia's modification of Wright's stain (11), diluted carbol fuchsin (16), Löeffler's methylene blue (18), gentian violet (19), Cross' stain (3), Benian's "relief stain" (1), and Burri's method (2). Spirochetes from cultures were somewhat more difficult to stain. Fontana-Tribondeau, Kliewe, and Kliewe-Harris were found most satisfactory and were used regularly. Various modifications of these stains were tried from time to time but no noticeable improvement over the original methods was observed.

In the examination of fixed stained material, only 2 types were recognized, the treponemes and spironemes. The fusi-spirals appeared similar to the spironemes when stained. They were usually S-shaped and contained one complete shallow spiral.

The treponemes and spironemes found in the mouth, caecum, and large intestine were indistinguishable morphologically, either under the dark-field or in fixed stained specimens.

2. Filtration and cultivation experiments

A. Filtration

Fourteen out of 18 filtrations by gravity and all of the filtrations with pressure of 6 pounds yielded spirochetes. The gravity filtrations were centrifuged at low speed for 5 to 10 minutes, the supernatant fluid decanted and the few remaining drops used. The spironeme was the only type of spirochete seen in the filtrates. In several filtrations with the Berkefeld N candle the spironemes alone came through, no bacteria appearing in the dark-field specimen. In the filtrations with the Berkefeld V under pressure a few bacteria were seen. When the filtrates were cultured as described below all contained bacterial contaminations. Filtered spirochetes were used in the cultivation experiments. When

filtrations were done with pressure the first few drops of the filtrate were used for cultivation and only after preliminary dark-field examination to exclude so far as possible the presence of bacteria.

B. Cultivation

Summarizing the results of approximately 600 tubes of fluid and semi-solid media inoculated with mouth, caecal, or intestinal material, 138 tubes were found upon examination to show greater or less increase of the spirochetes. All successful cultures were grown aerobically at 37°C. in media with a pH of 7.0.

Good growth of caecal and intestinal strains was obtained in Noguchi's diluted fluid medium (13) containing fresh cat liver and autoclaved rabbit liver, 3 successive subcultures being made from tubes of this type. The spirochetes were never obtained in pure cultures.

The mouth spirochetes did not multiply in this medium. A mixture of 5 cc. ascitic fluid and 5 cc. distilled water was found to give the greatest increase in number of mouth and intestinal spirochetes in the shortest time (approximately 2 days). Successful subcultures were also made in this medium. Hogue's ovomucoid medium (7) yielded growth of spirochetes from the mouth, caecum, and large intestine. Subcultures were also obtained in these cases. Noguchi's soft, semi-solid medium (14) was especially good for growing spirochetes from the mouth of the cat.

3. Implantation experiments in young cats per os and per rectum

Young cats 3 to 15 days old were used for the implantation experiments, because preliminary examination had shown that animals younger than 2 months did not have spirochetes in the mouth or gastrointestinal tract. Five groups of animals were used, lots of 3, 4, 3, 3, and 4 kittens constituting the 5 groups which were 13, 14, 15, 4, and 3 days old respectively when the experiments began.

Introduction of spirochetal material extended over a period of 2 weeks. The first 3 groups were fed the emulsified scrapings of the large intestine containing spirochetes of all types. The fourth group was fed the emulsified mucosal scrapings and diluted caecal contents of a hooded rat containing spirochetes morphologically similar to those found in the caecum and large intestine of the cat. In the fifth group half were given mouth spirochetes *per os* and half *per rectum*.

Summarizing the results, both mouth and intestinal types were implanted in the caecum after feeding *per os*. Mouth types were also

implanted in the caecum after introduction *per rectum*. A heavier implantation was secured when a preliminary dose of saturated solution of sodium sulfate had been administered. The mouth, liver, lung, heart, kidney, and spleen contained no spirochetes.

SUMMARY AND CONCLUSIONS

1. Forty-eight out of 50 normal adult cats contained spirochetes in the mouth or gastro-intestinal tract or both. Five out of 22 normal kittens, ranging in age from mature embryos to approximately 90 days, contained spirochetes in the caecum and large intestine. The animals containing these organisms were 70–90 days old.

2. The usual types of spirochetes observed were spironemes, treponemes, and fusi-spirals, present in large numbers in the alimentary tract. The first two types were abundant in the mouth, caecum, and large intestine. The fusi-spirals, while present in the mouth and large intestine, were most abundant in the caecum. Spironemes or treponemes or both were occasionally encountered in the duodenum, jejunum, or ileum. Spiral organisms with rigid, coarse, blunt spirals were found in a few stomachs. Leptospiras in relatively small numbers were also present at times in material from the mouth, caecum, and large intestine.

3. The most common type of spironeme in mouth or intestinal tract was approximately 10μ long and 0.5μ thick having 4 or more spirals about the same depth in the middle as at the ends with a depth of 0.6μ to 1.0μ and an amplitude of 1.0μ. A spironeme was also noted in material from the mouth of the cat which we regard as a new species and for which we suggest the name *Spironema langei*. It is a typical spironeme approximately 10μ long and 0.3μ to 0.5μ thick, with 3 to 6 spirals which were fairly constant when the organism was in motion and maintained their form in fixed preparations. The spiral depth and amplitude was 2μ. The ends were pointed and no terminal filaments were seen. A small projection occurred at the apex of each rounded spiral, clearly visible under the dark-field and in the films stained by the Kliewe-Harris method (Fig. 5). The fusi-spirals were 6μ to 10μ long, 0.5μ to 0.75μ thick in the center and tapering toward the ends which were sharply pointed. The organism when stained was S-shaped, usually contained one complete shallow spiral. The leptospiras were approximately 10μ long, very slender, with closely-set spirals and very flexible hooked ends. These types occurring in the mouth, caecum, and large intestine were morphologically indistinguishable.

4. The heart, lungs, liver, spleen, and kidney of 86 apparently normal cats and kittens did not contain spirochetes.

5. Cats younger than 2 months did not show spirochetes in the mouth or gastro-intestinal tract. Under natural conditions the spironeme was the first to appear in young cats.

6. All the organisms were satisfactorily stained. The Fontana, Kliewe, and Kliewe-Harris methods were the most satisfactory. Very delicate treponemes staining with more difficulty than *Treponema pallidum* were well demonstrated by the Kliewe-Harris method.

7. Spironemes from the caecum and large intestine were filtered through Berkefeld N and V candles by gravity in 5 to 6 hours, and by a pressure of 6 pounds in 5 to 10 minutes. The filtrate from the Berkefeld N candles was apparently free from bacteria when examined under the dark-field.

8. Growth was obtained in various media though the spirochetes were never obtained in pure culture. Successful cultivation of spirochetes from the mouth, caecum, and large intestine through two subcultures was obtained in 5 cc. ascitic fluid diluted with an equal amount of distilled water, both raw and filtered material being used. Successful cultivation with 3 subcultures was also obtained with diluted ascitic fluid (1:3), containing a piece of autoclaved rabbit liver, and in Hogue's ovomucoid medium. They were successfully grown in Noguchi's fluid medium but did not grow in the diluted ascitic fluid with liver tissue. All successful cultures were incubated aerobically at 37°.

9. Spirochetes were successfully implanted in kittens 5 to 15 days old, mouth spirochetes of the adult cat being implanted *per os* and *per rectum* and those from the large intestine *per os*.

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EXPLANATION OF PLATES

PLATE 21

Fig. 1. Spironeme No. 1. Short and longer forms. Thicker forms, Spironeme No. 2, also present. Source, mouth. Photomicrograph × 900.

Fig. 2. Spironemes No. 1 and No. 2. Spironeme (lower right) in process of transverse division. Source, mouth. × 900.

Fig. 3. Spironeme No. 2. Short and longer forms. Spironemes No. 3 (center) faintly stained. Source, mouth. × 900.

Fig. 4. (a) Zuelzer's *Spirochaeta pseudorecurrentis*. Handbuch der Pathogenen Protozoen 11: 1670. 1925. × 900.
(b) Spironeme No. 3. Spirochete from mouth of cat similar to Fig. 4a, in living material—spirals irregular when stained. × 900.

Fig. 5. Spironeme No. 4. *Spironema langei*, diagrammatic representation. Source, mouth.

Fig. 6. Knospen. Meirowsky, Med. Klin. **12**: 1181. 1916. Photomicrograph. × 900.

PLATE 22

(Photomicrographs × 900)

Fig. 7. Knospen on Spironeme from mouth of cat, similar to No. 53 of Fig. 6.

Fig. 8. Treponemes A and B, short and longer forms. Source, mouth. Spironeme No. 1.

Fig. 9. Treponemes A and B. Thicker forms of treponemes showing incurvation.

Fig. 10. Thicker forms of treponemes.

Fig. 11. Fusi-spirals.

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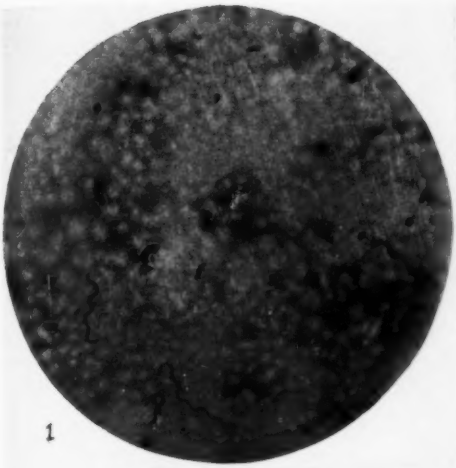
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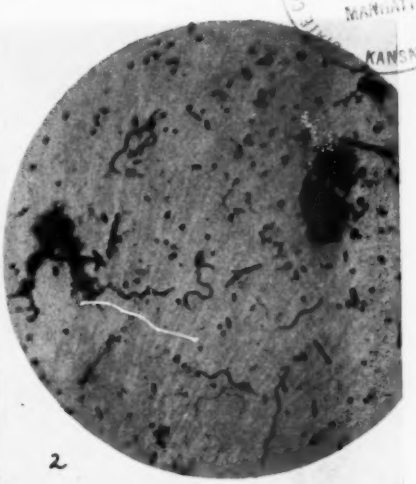
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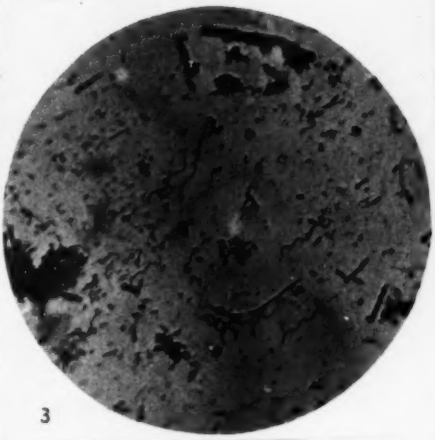
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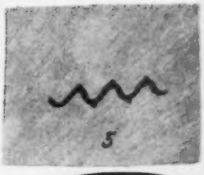
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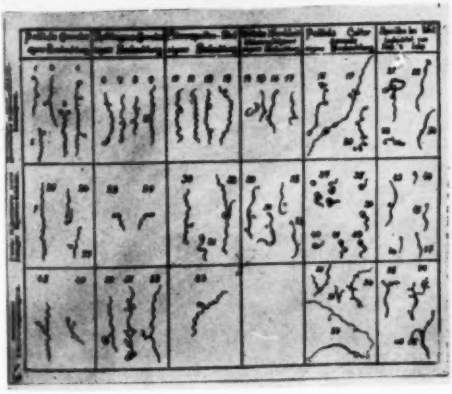

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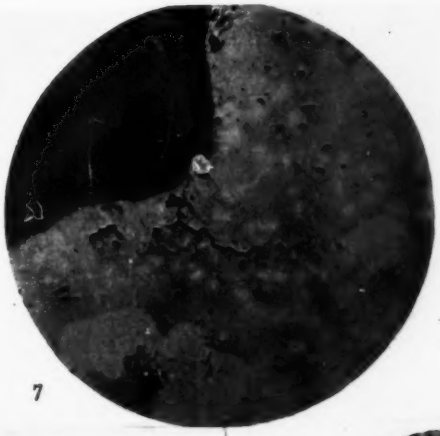

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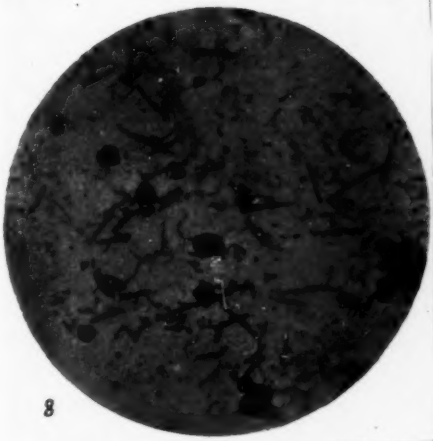

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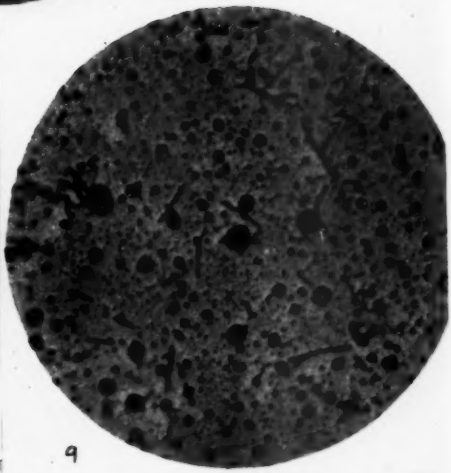

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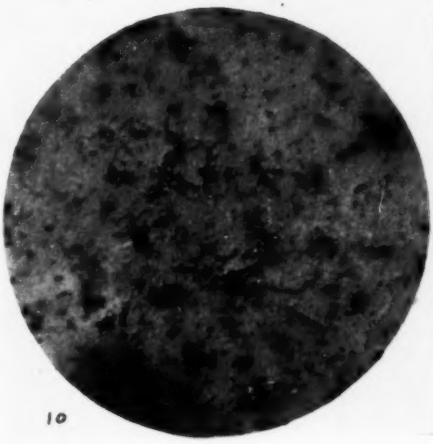

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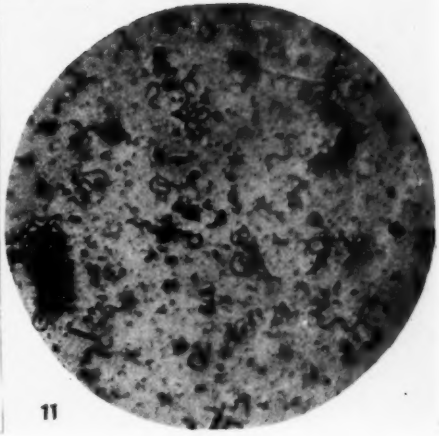

11

THE LIFE HISTORY OF POLYPODIUM POLYPODIOIDES HITCH., ESPECIALLY SPERMATOGENESIS[1]

By Douglass E. Rankin

Plates 23–26

INTRODUCTION

The following study of the gametophyte of *Polypodium polypodioides* was carried out in search of a better understanding of the behavior of the nucleus, the plastids, and mitochondria during the several phases of development of this fern.

This work includes a general study of the development of the antheridium, of the archegonium, of fertilization, and of the development of the embryo. A cytological study of spermatogenesis, carried on not only with nuclear fixatives but also with cytoplasmic and mitochondrial ones, is likewise included.

HISTORICAL SURVEY

The structure and development of the antheridium and archegonium of the Leptosporangiate ferns have been extensively studied. A rather complete bibliography of the subject up to 1923 has been published by Bower (7) in his book on the Filicales and need not be repeated here. More recent work on these phases in certain Leptosporangiatae has been done by Miss Stokey (31) on the Cyathaceae and by Miss Hartman (16) on the opening of antheridia in certain Polypodiaceae.

Comparatively few workers have studied fertilization in the ferns. Studies have been published by Strasburger (32) on *Pteris* and *Ceratopteris*, by Campbell on *Pilularia* (9) and on *Osmunda* (10), by Shaw (29) on *Onoclea*, by Thom (34) on *Adiantum* and *Aspidium*, and by Yamanouchi (37) on *Nephrodium*.

As mentioned by Yamanouchi (37), the two features of especial interest recorded in the above papers are:

1. The presence of a wall around the egg after fertilization.
2. The frequently collapsed condition of the egg after fertilization.

Strasburger (32) has stated that more than one sperm is prevented

[1] Botanical Contribution from the Johns Hopkins University No. 126.

from entering the egg by the formation of a heavy enclosing wall about this immediately after fertilization by the first one. Shaw (29) on the other hand believes that a partial collapse of the egg cell occurs after one sperm has entered. In this condition additional sperms are not able to enter. Mottier (20) doubts that the above condition is normal.

The development of the embryo of the Leptosporangiatae has been studied by Atkinson (2), Campbell (10), Leitgeb (19), Shaw (29) and, more recently, by Cross (11). The occasional formation of more than one embryo on a prothallus was noted by Atkinson (2), Etter (14), and Mottier (22).

Strasburger (32 and 33), Belajeff (3, 4, 5, 6), and Shaw (30) made important studies of the spermatogenesis of ferns during the latter part of the nineteenth century. A question under debate with most of these workers was whether or not the blepharoplast is homologous with the centrosome. Strasburger (33), from his work on the zoospores and gametes of the algae and on the spermatozoids of ferns, concluded that the cilia on both arise from the blepharoplast and that these structures are morphologically distinct from centrosomes. Belajeff (5) was the earliest worker to describe the development of the blepharoplast in Pteridophytes. He first saw the cilia arising from the blepharoplast in the spermatozoids of *Equisetum*. The work of Belajeff (5) and Shaw (30) on the spermatogenesis of *Marsilea* led both to conclude that the blepharoplasts in that plant are identical with centrosomes. Webber (35) and Ikeno (18) worked on the spermatogenesis of the Cycadaceae. Webber believed the cilium-bearing organ to be quite distinct from the centrosome and it was he who named the former the "blepharoplast." Ikeno, however, believed the two organs to be identical.

In discussions of the morphological development of plant spermatozoids, the interest until recently has been centered in the metamorphosis of the nucleus and of the cilium-bearing organ. Of late, however, there has become evident an interest in certain cytoplasmic elements other than the blepharoplast; namely, the chondriosomes, the plastids, and the vacuoles. The part that these structures play in spermatogenesis is still a matter of uncertainty and of controversy.

Yamanouchi (37), in his study of the spermatogenesis of *Nephrodium molle*, found that the blepharoplasts are first discoverable at the resting stage before the last nuclear division in the antheridium. At this time they appear as two darkly staining bodies, one on either side of the nucleus. They remain near the pole of the spindle during the division but no asters are found around them. When the spermatids are formed

one of these bodies is seen in each cell some distance from the nucleus. At the same time there appears opposite the blepharoplast a smaller body which is called the Nebenkern. The blepharoplast applies itself to the nuclear membrane and elongates along with the nucleus, following half way around the two and a half times coiled spermatozoid. The Nebenkern remains in the cytoplasm at the side of the nucleus and is finally found in the vesicle attached to the lower end of the spermatozoid.

Certain of these same structures were found by R. F. Allen (1) in her work on spermatogenesis in ferns. She did not, however, discover blepharoplasts in the spermatid mother cell nor find a structure comparable to the Nebenkern. On the other hand she did, like Buller (8), find starch in the vesicle of the spermatozoid, a fact which earlier workers had noted but which more recent investigators had not mentioned.

In *Equisetum*, Sharp (27) found a single centrosome surrounded by astral radiations in the penultimate generation of the antheridium. This divides to form the blepharoplasts which pass, with their asters, to opposite sides of the nucleus and occupy the poles of the spindle. In the spermatid the blepharoplast becomes vacuolated and fragments. Later these parts unite to form the cilium-bearing band. This band becomes associated with the nucleus in forming the spermatozoid but is not so closely bound to it as is the case in the ferns. He also found a series of darkly-staining bodies along the concave surface of the nucleus, the origin or nature of which he did not determine.

In a study of the spermatogenesis of *Marsilea* also, Sharp (28) decided that the blepharoplast is of the nature of a centrosome. In the first mitosis of the spermatogenous tissue the two centrioles are weakly developed but show astral radiations. In the second, third, and fourth divisions, however, they are very distinct. The blepharoplast becomes vacuolate and fragments as in *Equisetum*, but to a less degree. The parts here also later unite to give rise to the band which produces the cilia.

So little work has been done in all plants on the cytoplasmic elements other than the blepharoplast that the discussion of the literature on these subjects will be more general in nature.

Gavaudan and Cazalis (15) studied the behavior of the plastids in the Characeae during spermatogenesis. They found that the plastids retain their normal appearance until the spermatid begins its transformation into the spermatozoid. The plastids are then said to form a granular band on the posterior surface of the spermatozoid.

Motte (20), from his study of spermatogenesis in the mosses, con-

cluded that both mitochondria and plastids are present in the early stages of the development of the antheridium. In the later stages of spermatogenesis the plastids lose their starch and fragment. These fragmented plastids plus the mitochondria condense to form the solid limosphere which is attached to the posterior portion of the spermatozoid nucleus.

Weier (36) traced the development of the plastid through spermatogenesis in *Polytrichum* and *Catharinaea*. The young antheridia of these mosses, according to Weier, possess many plastids but their number becomes reduced by successive cell divisions until each androgone has only one plastid. This divides prior to each of the next two divisions. During the last division the plastid becomes reticulate. In the androcyte this reticulate body condenses to form the limosphere. The limosphere buds off the apical body and the remainder forms the vesicle at the base of the sperm. The apical body comes in contact with the blepharoplast which draws it to the anterior part of the sperm where it forms a slender pointed cytoplasmic filament.

In his work on the origin and evolution of the plastids in Pteridophytes, Emberger (13) discusses the spermatogenesis of *Adiantum*. He finds plastids and mitochondria present in the antheridium initials. The plastids become smaller and finally in the mother cell of the spermatid are hardly distinguishable from the mitochondria. Vacuoles filled by phenolic compounds are found in the early stages of the antheridium and are always present in protoplasts of the wall cells. These disappear by the time the spermatid mother cells are formed. In the spermatids there appear vacuoles, the nature of the contents of which was not determined. The granules at this time are indistinguishable except for a slight difference in size. There is, however, as Emberger thinks, a separation of the granules in the subsequent development of the spermatozoid. The plastids migrate to the vesicle and the mitochondria form a double row of granules along the length of the spermatozoid.

Dracinschi (12) studied the mature sperms in several Eufilicineae, and in *Pilularia* of the Hydropteridineae. She discusses the structure of the sperm under the headings of the cilium-bearing band, the nuclear substance, and the plasma material. The cilium-bearing band and nucleus form the spiral body of the sperm. The cilium-bearing band is broad at the anterior end and tapers toward its base. It is somewhat more than half the length of the spermatozoid. It bears three rows of cilia on the anterior end and the number of rows decreases to one as the band becomes smaller. Each cilium possesses a small, expanded, basal

portion and a stiff shaft about two microns in length. The remainder of the cilium is flexible and fibre-like in character. A darkly-stained zone which she calls the *Randsaum* (or marginal hem) runs along the outer anterior edge of the sperm. This, according to Dracinschi, is the structure that Belajeff (5) thought gave rise to the cilia and is what Shaw (30) later mistakenly called the blepharoplast. The nucleus is covered with a thin cytoplasmic sheath. In this sheath, on the outer, posterior surface of the spermatozoid, are found plate-like bodies which Dracinschi thought to be Golgi material. Chromomeres are found in the nucleus. Adhering to the inner margins of the coils of the nucleus is a row of granules, the morphological nature of which is not known but which Dracinschi thought might be of either plastid or vacuolar nature.

Yuasa (38) also studied the mature sperms of Leptosporangiate ferns. He found that in *Pteris cretica* the cilium-bearing band and the marginal hem both run the whole length of the sperm.

MATERIALS AND METHODS

The prothallia used in the following experiments were obtained from spores of *Polypodium polypodioides* collected near Fayetteville, North Carolina. The spores were sown on powdered peat which had been sifted and boiled. The spores germinated in about ten days. Antheridia were formed after eight weeks and archegonia in approximately ten weeks.

Bouin's fixation was employed for fixing the prothallia used in the morphological study of the development of the sexual organs, of fertilization, and of the development of the embryo. The two most successful stains employed were Heidenhain's iron haematoxylin for the chromatin of the nuclei and Delafield's haematoxylin for the cell walls.

For the work on spermatogenesis various other fixing agents and stains were employed to bring out the different cell components. Flemming's weaker fixative followed by Heidenhain's haematoxylin was used for the blepharoplast and nucleus. Prothallia fixed in Bouin's mixture, after pre-fixation with ten per cent formalin neutralized with $MgCO_3$, gave striking pictures of the blepharoplast and also of the development and the various phases of structure of the nucleolus. The chromosomes were fixed by this technique, but the chromatin reticulum was not stained. Champy-Kull's fixative and Altmann's stain fixed and stained mitochondria, plastids, blepharoplasts and nucleoli. By this method the blepharoplast and nucleus of the mature spermatozoid were differ-

entiated; other combinations failed to show this. The mitochondria when fixed with Regaud's medium and stained with Heidenhain's haematoxylin were seen more distinctly than by any other method. The mitochondria in the mature spermatozoid were especially clear. Starch in the mature spermatozoid stained purple with gentian violet after Merkel's fixative. It was also stained in the spermatid and spermatozoid with Gram's iodine stain, following fixation by Regaud's method. Several other fixatives and stains were used but gave no essentially different results and hence will not be discussed here. Mann-Kopsch's fixing liquid showed the development of plastids; Bouin's fixative, with Feulgen's reaction as a stain, was used for the chromatin. Vital stains such as neutral red, methylene blue, and Janus green were employed also, but without satisfactory results.

MORPHOLOGICAL STUDY OF THE REPRODUCTIVE ORGANS, FERTILIZATION, AND THE DEVELOPMENT OF THE EMBRYO

Development of the prothallus. The first sex organs to appear on prothallia of *Polypodium polypodioides* are the antheridia. These occur on plants which are about two months old and are formed before the prothallium has become heart-shaped, or at least soon after the notch appears. The antheridium-bearing plants are small and one or more cells in thickness (figs. 1, 2). In some cases the antheridia cover practically the entire upper and lower surfaces of the prothallium, leaving only a narrow margin of sterile vegetative cells across the apical end. Such plants apparently never form archegonia. Other male plants which have not borne such a heavy crop of antheridia continue vegetative growth for some time and then form archegonia.

The first archegonia were formed on prothallia of the same sowing as that last mentioned which had *not* borne antheridia. They developed about two weeks after antheridia had appeared. The female plants were more characteristically heart-shaped and about three times as large as the male plants of the same culture. They possessed the typical archegonial cushion along the central portion of the prothallium. The archegonia are formed on this cushion from both the upper and lower surfaces. The archegonia form on the lower surface first, as is also true of antheridia. Only rarely were both types of reproductive organs found on the same plant at one time. When this did occur the antheridia were formed on proliferations from the margins or cushions of plants the bodies of which had previously borne no antheridia but archegonia only. A culture of *Polypodium polypodioides* about four months

old is shown in figure 3. This photograph shows the archegonial plants distinctly but the smaller, antheridial prothallia are not clear.

The development of the antheridium. The development of this organ resembles that found in many other Polypodiaceae. A prothallial cell enlarges and divides at an often slightly oblique angle, to give rise to a basal cell and an antheridium initial (fig. 42). The first division in the initial produces a cup-shaped cell whose rim touches the lateral wall of the antheridium initial and whose base may or may not come into contact with the upper wall of the basal cell (fig. 22). The second wall is concentric with the upper surface of the antheridium and intersects the first wall in a ring (fig. 22). In this upper cell is formed the third wall, a circular anticline, which cuts out a disk-like cover cell with sloping sides (fig. 23). It is this latter cell which, by bursting, or by being dislodged intact, allows the spermatozoids to escape. The three cells so formed surround a large central cell, which is the primary spermatogenous cell. In the central cell the first division is longitudinal and the second one transverse to the axis of the antheridium. After this one, two, or rarely three more divisions may occur, giving respectively 8, 16, and 32 spermatids. In some of the smaller prothallia none of the antheridia had more than eight spermatozoids. Generally, however, about half of the antheridia of the smaller plants produced eight spermatozoids each and half produced sixteen spermatozoids. On the larger prothallia, on the other hand, about one-third of the antheridia produced eight spermatozoids, and two-thirds produced sixteen. Sometimes a slightly larger proportion of the antheridia produced sixteen spermatozoids each, i.e. larger prothallia have larger antheridia. Antheridia with thirty-two spermatozoids were rare.

The mode of opening of the antheridium corresponds with that described by Miss Hartman for certain other Polypodiaceae. The cover cell, though it is occasionally extruded intact, is more often burst and its contents form a granular mass.

Development of the archegonium. The archegonia arise in acropetal succession on the cushion region of the prothallium. The initial cell of the archegonium is formed by a division of a superficial cell in this region (fig. 4). It can be identified by its large nucleus and by its cytoplasm which is denser than that of the surrounding vegetative cells.

When the archegonium initial is formed it undergoes two successive transverse divisions, giving rise to the basal cell, the central cell, and the primary neck cell (fig. 5). The central cell is, like the archegonium initial, conspicuous because of its large nucleus and dense cytoplasm.

The first division in the young archegonium is in the primary cover cell (fig. 6). The cell wall so formed is an anticline parallel to the axis of the archegonium. The two neck cells immediately undergo another anticlinal division perpendicular to the first. Thus are formed the four neck cells that are present at this time in the archegonium. The central cell, meanwhile, has started to enlarge. As it increases in size the neck cells divide by anticlines transverse to the neck and so keep pace with it, and in this manner is formed a neck which projects beyond the surface of the prothallus.

The first division in the central cell is transverse (fig. 7). This division gives rise to a large inner cell which is embedded in the vegetative tissue of the prothallus and a smaller primary neck canal cell that is surrounded by the wall cells of the neck which project beyond the prothallus. The inner or ventral cell divides before the primary neck cell. When the spindle for this division is formed it is situated in the outer portion of the cell (fig. 8). Thus when the wall is formed the two resulting cells are of quite unequal sizes. The larger inner one is the egg and the smaller outer one is the ventral canal cell. Following this division in the ventral cell a nuclear division takes place in the primary neck canal cell. This is also a transverse division. Most often a cleavage of the cytoplasm occurs, but no wall is formed between the two nuclei which are produced by this division (fig. 10). In some few cases, however, a wall may occur.

Thus the mature archegonium contains a large egg cell, a smaller ventral canal cell, and two neck canal cells or nuclei. As the archegonium matures the egg cell becomes indented due to pressure of the three canal cells above it (fig. 9). Then too, as the archegonium becomes older the two nuclei of the neck migrate to the outer end of the neck and become packed tightly together giving them, superficially, the appearance of being fused.

When prothallia bearing these mature archegonia are watered, the cells of the neck become more turgid and the inner cells swell. As a result the archegonium is ruptured at the apex. This leaves a chimney-like neck with an opening above, through which a slimy substance is discharged. The slimy substance is evidently a disintegration product of the three canal cells. The egg cell which was indented before the opening of the archegonium becomes rounded when pressure is released by the discharge of part of the contents of the neck.

Fertilization. The spermatozoids are attracted to the neck of the open archegonium. Pfeffer (26) and Hoyt (17) have noted that this

is due to the presence of malic acid or malates in the disorganized contents of the ventral canal and neck canal cells. Many spermatozoids were observed in the neck of the archegonium but never more than one could be found to have penetrated the egg (fig. 12). After fertilization eggs often presented a collapsed appearance, though other eggs, also containing spermatozoids and in approximately the same stage of development, were well rounded. No unusually heavy wall was observed around the recently fertilized eggs. The spermatozoid while in the neck of the archegonium is very much elongated and loosely coiled (fig. 11). When, however, it has entered the egg the spermatozoid condenses to form a thick, coiled body (fig. 12). The chromatin of the egg nucleus during the penetration of the sperm forms a coarse net. The sperm nucleus next becomes reticulate (fig. 13). Soon the reticulum of the male nucleus becomes indistinguishable from the reticulum of the egg nucleus.

Development of the embryo. The fertilized egg enlarges greatly during the resting stage preceding the first division (fig. 14). The first wall formed is slightly oblique to the neck of the archegonium and transverse to the long axis of the prothallus (fig. 15). This divides the embryo into anterior and posterior halves. The second wall is transverse to the neck of the archegonium and parallel to the long axis of the prothallus (fig. 16). The embryo is thus divided into quadrants. The next wall is the octant wall which is parallel to the neck of the archegonium and in the plane of the long axis of the prothallus. Two each of these octants are used in the formation of the primary organs of the plant. In an embryo developed on the lower surface of the prothallus the cotyledon is formed in the lower anterior segments, the stem from an upper anterior segment, the root from a lower posterior segment and the foot from the upper posterior segments. The walls developed within the octants are somewhat irregular. The first walls, however, are nearly always parallel to the anticlinal octant walls (figs. 17 and 18).

The initials are distinguishable even in embryos as young as that shown in figure 18. An older stage showing the root, stem, and cotyledon initials is illustrated in figure 19. In this embryo the quadrant walls are also distinct. A still older embryo in which the root and cotyledon have elongated is shown in figure 21.

CYTOLOGICAL STUDY OF SPERMATOGENESIS—STRUCTURE OF THE SPERMATID AND THE SPERMATOZOID AS DETERMINED BY THE USE OF SEVERAL CYTOLOGICAL METHODS

1. Results with Flemming's fixative and Heidenhain's haematoxylin stain

In material fixed and stained by this combination of methods the chromatin and the blepharoplasts are dark blue. This is an excellent fixative and stain for these elements and their development can easily be demonstrated with it. The central cell has a large nucleus and one or two large and often irregular nucleoli (fig. 22). With this technique the nucleoli are distinguishable up to, and sometimes after, the development of the spermatids but never show as distinctly as with the two methods to be described next.

The blepharoplasts are first discoverable in the resting stage of the spermatid mother cell (fig. 24). They appear as two darkly staining bodies, one lying on each side of the nucleus at some distance away from the wall of the latter. The chromatin of the nucleus then forms a coarse reticulum with distinctly thicker and thinner portions. At prophase the blepharoplasts move still farther away from the nucleus and occupy the poles of the spindle, but show no astral radiations (fig. 25). The metaphase, telophase, and anaphase were not found in cells fixed and stained by this method. Cells fixed by Champy-Kull's method showed the blepharoplast during the metaphase. During the resting stage of the spermatid nucleus the blepharoplast appears as a very conspicuous black body lying in the cytoplasm at some distance from the nucleus and the chromatin of the latter appears as a dense network (fig. 26). The blepharoplast approaches the nucleus and finally becomes applied to the nuclear membrane (fig. 27). At this stage it begins to elongate (fig. 28). When the blepharoplast has extended a little over half way around the still globular nucleus, the latter begins to change its form and inner structure. The threads of the chromatin network become more and more thickened (fig. 29) while the side of the nucleus opposite the blepharoplast becomes at first flattened and finally concave (fig. 30). During the time the nucleus is thus crescent shaped in lateral view the blepharoplast *cannot be differentiated with haematoxylin.* When the chromatin has condensed to form the more attenuate, coiled spermatozoid nucleus, however, the blepharoplast can be shown, extending approximately one coil beyond the nucleus, which itself shows from one and one-fourth to one and one-half turns (fig. 31).

2. Results obtained with neutral formalin, Bouin's fixative, and Heidenhain's haematoxylin stain

This method fixes and stains nucleoli, blepharoplasts, and the chromosomes. It does not stain chromatin when in the reticulum stage. The central cell shows one or two large, and often irregular, nucleoli (fig. 33). In the two-celled stage some cells were found with one nucleolus, some with two nucleoli and some with nucleoli in the process of division by constriction. Nucleoli in one or the other of these three phases were found in the resting nuclei of all stages up to the time the spermatid nucleus began its metamorphosis into a spermatozoid. The following tables show the combinations of those cell stages and nucleolar phases that were actually found:

(1) Four-celled stage.
  (a) Each of the four cells of the antheridium contained one nucleolus.
  (b) Each of the four cells of the antheridium contained two nucleoli.
  (c) Three cells of the antheridium contained two nucleoli and one had a dividing nucleolus.
(2) Eight-celled stage.
  (a) Each of the eight cells of the antheridium contained one nucleolus.
  (b) Five of the eight cells of the antheridium contained two nucleoli and three cells contained one nucleolus each.
  (c) One cell of the antheridium contained two nucleoli and seven cells contained one nucleolus each.
(3) Sixteen-celled stage.
  (a) Each of the sixteen cells of the antheridium contained one nucleolus only.
  (b) Each of the sixteen cells of the antheridium contained two nucleoli.

This technique makes the blepharoplasts even more conspicuous than does Flemming's, and the same developmental stages of these were found.

3. Results with Champy-Kull's fixative and Altmann's stain

These fix and stain blepharoplasts, mitochondria, plastids, and nucleoli distinctly, but stain chromosomes only faintly. In addition to the nucleus and the plastids this method demonstrates the presence in the prothallial cells of certain small purple-staining granules. In the prothallial cell at the mitotic division preceding the formation of the antheridium initial the mitochondrial granules, which have increased in number, are grouped about the poles of the spindle (fig. 42). Thus, when the initial of the antheridium is formed, it contains the plastids

which were in the outer part of the prothallial cell and the mitochondrial granules which surrounded the daughter nucleus. In the wall cells there are numerous plastids, smaller in size than those of the prothallial cells. The mitochondria in the wall cells are more numerous than in the prothallial cells but not as abundant as in the spermatogenous cells. These latter cells, on the contrary, though they have many more mitochondria, have fewer and smaller plastids than the wall cells (fig. 43). The plastids in both can be recognized by their deeply-staining outer portion and lightly-staining central region. The cells resulting from the divisions up to the spermatid mother cell stage are essentially alike. The mitochondria are very numerous and grouped about the poles during mitosis. The plastids up to this spermatid mother cell stage are still easily distinguishable. The one or two nucleoli of each cell stain faintly.

With this technique the blepharoplasts appear at metaphase of the division of the spermatid mother cell, as two conspicuous red bodies in each cell (fig. 46). The spermatid at its formation contains numerous mitochondria scattered through the cell (fig. 47). It also contains a large blepharoplast located at some distance from the nuclear membrane. The development of the blepharoplast has been described above. With other techniques, however, it was not distinguishable in the mature spermatozoid. With Champy-Kull's method the red stained blepharoplast can be seen to extend beyond the purple stained nucleus and is applied to a relatively small part of the upper portion of the nucleus (figs. 50, 51). As the nucleus begins to coil, preparatory to forming the spermatozoid, the mitochondria seem to become more closely associated with it (fig. 49). In the mature spermatozoid they form a double row of granules on the inner surface of the coiled nucleus (figs. 50, 51). This condition will be discussed in greater detail when recording the results obtained with Regaud's technique. The plastids were not distinguishable with the Champy-Kull method after the nucleus began to coil.

4. Results with Regaud's fixative and Heidenhain's haematoxylin stain

This method fixes and stains both mitochondria and plastids. Mitochondria fixed by this technique are vastly different in appearance from those fixed with Champy-Kull's. Fixed with Regaud's fluid and stained with Altmann's stain they appear as short rods and as when fixed with Champy-Kull are far more numerous in the central cell than in the wall cells. The plastids, which are about equal in length to the mitochondria, are distinguishable from them by their heavily staining outer portion

and lightly-staining interior. The rod-like type of mitochondrium is present up to the formation of the spermatid. In the spermatid the mitochondria appear either as short rods (fig. 54) or as longer rods forming a more or less broken chain (fig. 58). At this stage the plastids also are still distinguishable (fig. 56). As the nucleus begins to coil, the rod-like mitochondria are found along its edges and scattered over its surface (figs. 59, 60). As the nucleus coils more and more the mitochondria become arranged in two rows, like two strings of beads, one along each edge of the nucleus on the inner surface of the coil and then extend to form a single row along that portion of the blepharoplast which protrudes beyond the nucleus (figs. 61, 62, 63).

DISCUSSION

Morphological study of Polypodium polypodioides

In studying the development of the prothallus of *Polypodium polypodioides* several problems of interest arose. It was found that antheridia and archegonia are produced in almost equal abundance on the upper as well as the lower surface of their respective prothallia. Reproductive organs appear first on the lower surface but soon form on the upper surface also. Another point of interest was the fact that archegonial plants very commonly formed proliferations from the margins and cushion of the prothallia. Mottier (23) has described these from cultures which he kept growing for many years. In *Polypodium polypodioides* they occurred on prothallia which were about six months old and which had apparently been exposed to no adverse conditions.

In the development of the archegonium it was found that sometimes a wall is formed between the two nuclei of the neck canal cell. This was noticed by Campbell (10) also in *Osmunda*. This condition in both *Osmunda* and *Polypodium polypodioides* is a rare occurrence.

The factor which prevents more than one sperm from entering the egg is as yet undetermined. Shaw (29) thinks that it is accomplished by the collapse of the egg after the sperm has entered. Mottier (21), on the other hand, thinks this collapsed condition is due to fixation. Strasburger (32) said that a heavy wall formed around the egg immediately after fertilization, and that this prevented additional sperms from entering. It was observed in *Polypodium polypodioides* that a few eggs which contained spermatozoids were collapsed. Many other eggs containing spermatozoids, in apparently the same stage, were turgid. Two conclusions might be drawn from this. Either the collapsed state of the recently fertilized egg is due to fixation or it is a normal process. If

it is normal the rounded fertilized eggs are older than the collapsed ones and have passed through that phase and later have regained their turgor. No unusually heavy wall was ever found around these eggs. Thus more evidence was obtained for Shaw's view than for the view held by Strasburger.

The development of the embryo of *Polypodium polypodioides* is much like that of other Polypodiaceae. Polyembryony, probably due to cultural conditions, was often found. As many as eight young embryos were sometimes found on a single plant and as many as three embryos on one plant might have one cauline leaf developed. This condition has been noted by Mottier (22) in certain Polypodiaceae and Osmundaceae.

CYTOLOGICAL STUDY

The nucleus and the blepharoplasts, as stated above in the review of the literature, are the objects which have received most attention in recent studies of spermatogenesis. The investigations of Belajeff (5, 6) and Shaw (30) on spermatogenesis in *Marsilea* and of Sharp (27) in *Equisetum* led them to conclude that the blepharoplasts in plant spermatozoids are morphologically the same as the centrosomes. They have also shown that the cilia arise from the blepharoplast. The work of Yamanouchi (37) on spermatogenesis in *Nephrodium* shows that the blepharoplast in that fern is lacking many of the characters of a centrosome. It does not divide to form daughter centrosomes, it only occasionally occupies the poles of the spindle and does not have astral radiations formed about it.

The blepharoplasts in *Polypodium polypodioides* first appear at the resting stage prior to the last division in the antheridium (fig. 24) as darkly-staining bodies, one on either side of the nucleus. During the last division they are found to occupy the poles of the spindle but no astral radiations are seen around them (fig. 25). When this division is completed and the daughter cells are formed there is found in each spermatid one large blepharoplast, situated in the cytoplasm at some distance from the nucleus (fig. 26). The chromatin of the spermatid nucleus at this time forms a coarse reticulum. The blepharoplast next approaches the nuclear membrane and finally comes in contact with it (fig. 27). After it has become applied to the nuclear membrane the blepharoplast begins to elongate. In the early stages of its elongation one end of the blepharoplast may be in contact with the nucleus while the other end extends out into the cytoplasm (fig. 28). As it elongates further, however, the blepharoplast extends half way around the still

globular spermatid nucleus. In preparations which show the coarse chromatin reticulum no nucleoli are seen. Other fixatives, however, brought out the fact that there are present in the spermatid one or two large nucleoli which are conspicuous until the nucleus begins to coil. After the blepharoplast has reached half way around the spermatid nucleus the chromatin begins to condense. The nucleus begins to flatten on the side opposite the blepharoplast and finally becomes concave (fig. 30). The chromatin continues to condense until it forms a solid, strap-like coiled body. The blepharoplast is applied to about one-third of the upper portion of the nucleus and extends one coil beyond the nucleus (fig. 31). This development of the blepharoplast and nucleus resembles that found in *Nephrodium* by Yamanouchi (37). Yamanouchi, however, does not find the blepharoplasts constantly located at the spindle poles during the last division, while in *Polypodium polypodioides* this does seem always to be true. Thus it appears that the blepharoplasts in this plant retain one centrosome-like character. The blepharoplast in this fern, as was also true in *Nephrodium*, is always a solid body. No segmentation occurs such as was found by Sharp (28) in *Marsilea* and *Equisetum* (27). The connections of the cilia with the blepharoplast were not seen nor could the writer find any division of the blepharoplast into a marginal hem and a cilium-bearing band such as Miss Dracinschi (12) describes.

The nucleolus. The changes in the nucleolus during spermatogenesis have not been discussed very extensively in most earlier studies of this stage. This is due to the fact that nucleoli are usually not very conspicuous in material fixed primarily for the study of chromatin. Yamanouchi (37) found either one or two nucleoli present in the spermatid of *Nephrodium*. And Sharp (27) in *Equisetum* figures from one to three nucleoli in the spermatogenous cells before their last division and but one nucleolus in the spermatid.

In *Polypodium polypodioides* the nucleoli stained very clearly with Heidenhain's haematoxylin after fixation with neutral formalin followed by Bouin's mixture. The central cell of the antheridium may contain one or two large, irregular nucleoli (fig. 33). In subsequent stages the nucleoli are smaller and more rounded. All nuclei in cells ranging from those in the two-celled antheridia to those of the spermatid contain either one nucleolus, a nucleolus in the process of constriction, or two nucleoli. For example, one four-celled antheridium was found in which three cells contained two nucleoli and one cell contained a nucleolus in the process of constriction. One eight-celled antheridium

was found with five nuclei containing two nucleoli and three nuclei containing one nucleolus each. Where there were two nucleoli in a nucleus these nucleoli were much smaller than that of a nucleus which had but one nucleolus. These facts give clear evidence that the nucleolar material in the antheridium of *Polypodium polypodioides* divides prior to nuclear division. The relation of the nucleolus to the chromatin reticulum has not thus far been studied since the neutral formalin rendered the chromatin unstainable. No trace of the nucleolus during mitosis was found.

Development of the plastids. In considering the development of the plastids in ferns it was thought advisable to review that phase of spermatogenesis in some other plants.

Gavaudan and Cazalis (15) studied the plastids in the spermatogenesis of the Characeae and found that the plastids containing carotin and starch stain distinctly up to the time that the spermatid nucleus begins to coil. At the time of the formation of the spermatozoid the plastids form a granular band on its posterior surface. A granular band was also seen in *Chara* by Muhldorf (25) who, however, found it to be composed only of starch.

Motte (20), working on spermatogenesis in the mosses, found a chondriome made up of long, narrow plastids and smaller mitochondria in the early stages of the antheridium. The plastids lose their starch and fragment to form, with the existing mitochondria, a granular mass which condenses to form a solid limosphere. In *Polytrichum*, Weier (36) found that one plastid remained in the androcyte, became reticulate and finally formed the limosphere. Then the limosphere, by division, formed the limosphere remnant and the apical body. The former contains starch and is found at the base of the spermatozoid; the latter is applied to the blepharoplast.

This work on the development of the plastids during spermatogenesis in the Characeae and in the mosses serves to emphasize several points. First, the behavior of the plastids during spermatogenesis varies in different groups. Second, the plastids in these groups have a complicated but regular developmental history during this process. Third, the plastids furnish the mature spermatozoid with the starch which is necessary to its functioning.

Yamanouchi (37), in his work on *Nephrodium*, did not discuss the early development of the antheridium. He does state, however, that the cytoplasm of the spermatid mother cell does not generally contain plastids. This was due, probably, to the fact that he used Flemming's

fixative which does not satisfactorily fix plastids except when they are mature.

Sharp (27), in his study of *Equisetum*, found plastids in various stages of disorganization up to the 8- or 16-celled stage and even in the spermatid. His interest at this time was, however, centered in the blepharoplast, hence in order to see these clearly he selected cells that contained the fewest plastids. The present work on *Polypodium polypodioides* does not indicate that the plastids disorganize but rather that they are present in all stages of spermatogenesis. The presence of these plastids could always be determined by their characteristic appearance during the early stages or in later stages by their contained starch.

Emberger (13) used Regaud's fixative in his study of spermatogenesis in ferns. He found that the wall cells decrease in size from losing their starch. The plastids of the central cell of the archegonium decrease in size until they are but slightly larger than the mitochondria. They can, however, be distinguished from the mitochondria by this slight excess of bulk. After the last division they form very small granules, practically indistinguishable from the mitochondria. A part of the granules migrate to the protoplasmic vesicle in the coil of the spermatozoid. He concludes that these bodies are the plastids since earlier workers, for example, Buller (8), found starch present in these vesicles. Recently, Muhldorf (24) also found starch in the protoplasmic vesicles of fern spermatozoids.

The plastids in the prothallia of *Polypodium polypodioides* are of the type described by Zirkle (39) for the higher plants. They are hollow, flattened spheroids. These plastids cover completely the exposed surfaces of the prothallial cells. They are composed of an outer chromatic stroma and an internal vacuole in which starch is stored. The plastids stain pink with Altmann's stain used after fixation with Champy-Kull's mixture. With this fixative and stain their development can be traced through all stages from the prothallial cells to the spermatid.

When the initial cells of the antheridium are cut off from the prothallial cells the plastids, which are found in the initials, are slightly smaller than those of the vegetative cells. The two divisions which follow in the initial cell divide that cell into an upper and lower wall cell and a central cell which is the primary spermatogenous cell (fig. 43). In the two wall cells the plastids are approximately of the same size as those of the antheridium initial. The plastids in the central cell on the other hand have decreased in size since they fail to grow after each division. They still possess, however, their characteristic structure, an outer

chromatic layer and an internal vacuole. Although the plastids vary in size, shape, and number in individual cells they are essentially alike in all cells of the spermatogenous tissue from the central cell to the formation of the spermatid (figs. 43–45).

After the spermatids are formed the plastids are no longer distinguishable in material fixed with Champy-Kull's fixative and stained with Altmann's stain. It was thus necessary to devise some method by which the presence of plastids in the spermatids could be detected. It was found after many unsuccessful attempts that material fixed in Regaud's mixture and stained by Gram's iodine method showed the presence of starch in the spermatid and in the spermatozoid. The starch was not noticeable in the younger stages of the antheridium with this method. It was then assumed that the starch had increased greatly in amount in the spermatid. These large starch grains in the plastids stretched the walls to such an extent that they could not be distinguished even after staining. Starch was also found in the spermatozoid when material fixed in Merkle's fixative was stained with Flemming's triple stain (fig. 52). With this method the starch in both the spermatozoid and the prothallial cells stained purple. The starch present in the spermatozoid is contained in the protoplasmic vesicle at the base of the spermatozoid.

From this work on the behavior of the plastids during spermatogenesis in *Polypodium polypodioides* it was found that the plastids play a definite rôle during this process. They can be identified in all cells before spermatid formation by size, structure, and staining reaction. After the spermatids are formed they can be identified by the starch which they contain.

The chondriome. There has been very little mention of the chondriome in connection with the discussion of spermatogenesis in plants, although it has long been known to be an important element in animal spermatogenesis.

In the mosses Motte (20) found that the mitochondria, and the fragmented plastids condense to form the limosphere. Weier (36) did not find any evidence of the chondriome in *Polytrichum*. He thought this might be due to some uncontrolled factor in the technique employed.

The only discussion of the behavior of the chondriome as such, during spermatogenesis in ferns, is that of Emberger (13). He finds that a few mitochondria are present in the antheridial initial and a larger number are present in the spermatogenous cells. When the nucleus of the spermatid begins to coil these granules are visible in the cytoplasm

surrounding it. In the mature coiled spermatozoid they are seen as two rows of granules along the inner surface of the coil. The mitochondria found in *Polypodium polypodioides* have essentially the same development and resulting position as that described by Emberger (13). There are points of variation which will be discussed later but these do not seriously interfere with the interpretation.

Two other workers, Sharp (27) and Miss Dracinschi (12), have described similar bodies in the mature spermatozoid. Sharp found a row of dark, rounded, bodies ranging, in *Equisetum*, along the inner surface of the coiled spermatozoid. He has mentioned them only to state that their nature and origin were not determined. Bodies of a similar appearance were also seen in the spermatozoid of the Eufilicinae and one of the Hydropteridineae, *Pilularia*, by Miss Dracinschi. She also could not explain their nature. The mitochondria in *Polypodium polypodioides* appear to be very similar to those described by Emberger (13) and those figured by Dracinschi (12) and by Sharp (27).

The mitochondria in *Polypodium polypodioides*, as has been stated above, differ somewhat from those of *Adiantum* described by Emberger (13). This difference is a matter of inconstancy in their shape, and in their size and arrangement at certain stages.

With Champy-Kull's fixative and Altmann's stain a few mitochondria are found to be present in the prothallial cells. They are short rods or granules which stain violet. They are found sometimes enveloping the plastids and sometimes in the cytoplasm between the plastids. During the division of the prothallial cell which forms the antheridium initial the mitochondria are found grouped about the spindle poles (fig. 42). In the initial cell of the antheridium the granules are more numerous than in the prothallial cells. The mitochondria are still more numerous in the wall cells of the antheridium than in the initial cells. However, they are fewer in number than in the central or primary spermatogenous cell. In this central cell there are exceedingly numerous purple granules, much smaller than the plastids, which are also present at that time (fig. 43). The mitochondria in all cells up to the formation of the spermatid have practically the same appearance as those of the central cell. The mitochondria are grouped about the poles of the spindle during the mitotic divisions of the spermatogenous tissue as was also true in the divisions which formed the antheridium initial.

When the spermatids are formed mitochondria are still abundant and scattered throughout the cytoplasm of the cell. The mitochondrial

granules begin to migrate toward the nucleus from the outer portions of the cytoplasm when the nucleus of the spermatid begins its metamorphosis. They can be observed scattered over its outer surface when the nucleus is partially coiled; however, they are usually more numerous along the margins of the coil (fig. 49). By the time the chromatin of the nucleus has condensed to form a nearly solid body the mitochondria are found as a double row of granules along the inner surface (figs. 50, 51).

Regaud's fixative gave two quite different results. In some material the mitochondria appeared as short rods in the prothallial cells, all the antheridial cells, and spermatids (fig. 54). The mitochondria grouped themselves about the metamorphosing nucleus and finally migrated to the under surface where they formed the two rows of granules. In other prothallia fixed with Regaud's fixative the mitochondria were seen to form a broken network in the antheridium initial instead of forming separate rods (fig. 53). This network was observed also in the different stages of the antheridium up to the last division of the spermatogenous tissue. In the spermatid this network was especially noticeable and while the nucleus is central these mitochondria seem to surround the nucleus on all sides (figs. 57, 58).

After the nucleus has condensed as in the preceding cases, the mitochondria form the double row of granules around the inner surface of the spermatozoid (figs. 61–63). Between the rod-like mitochondria and the mitochondria which formed the broken network there were many intermediate stages. It was thus concluded that there was either some slight variation in technique during different fixations or that the physiological condition of the prothallia varied. Either, it is believed, could account for a difference in the morphological character of the mitochondria.

The differences in the mitochondria as shown by Champy-Kull's and by Regaud's fixatives must be due to technique. The fact that in both cases the granules form a double row along the inner surface of the spermatozoid is proof of the fact that these are identical structures.

SUMMARY

Morphological. Antheridia appear on prothallia of *Polypodium polypodioides* when the plants are about two months old. Archegonia appear about two weeks later on other plants of the same sowing which are approximately three times as large as the male plants mentioned. Both types of organs occur on both upper and lower surfaces of the

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prothallia. Antheridia and archegonia occurred on the same plants simultaneously only where antheridia arose on proliferations of plants that had already borne archegonia.

The development of the antheridium and archegonium corresponds to that in many other Polypodiaceae. The opening of the antheridium is by the extrusion of the cover cell with wall intact or as a granular mass. In the archegonium, a wall is occasionally formed separating the two nuclei of the neck canal cells.

The ripe antheridia and archegonia open in a few minutes after the prothallia are watered. Many spermatozoids enter the open neck of the archegonium, but only one penetrates the egg. The egg is turgid before the penetration of the sperm. Many of the eggs observed immediately after fertilization were in a collapsed condition. No especially heavy wall was formed around the recently fertilized eggs.

Cytological. The blepharoplasts arise in the resting stage of the spermagones before the last nuclear division in the antheridium. They occupy the poles of the spindle during the subsequent mitosis. When the spermatids are formed one blepharoplast is found in each cell and is situated at some distance from the nucleus. The blepharoplast moves toward the coarsely reticulate nucleus and becomes applied to the nuclear membrane. Here it elongates until it has reached one-half way around the still globular spermatid nucleus. At this time the nucleus begins to condense and forms a strap-like coiled body. The blepharoplast, in a mature spermatozoid which is still inside the antheridium, has stretched to form about four-fifths of a complete coil and the nucleus forms about one and a half coils. The blepharoplast extends beyond the nucleus approximately half of a coil.

The structure and development of the nucleoli was traced, using material fixed in neutral formalin followed by Bouin's fluid. One or two large irregular nucleoli are found in the central cell of the antheridium. One or two nucleoli are also almost constantly found during the subsequent generations of the spermatogenous tissue. Oftentimes a constricted nucleolus was observed. These latter were seen even after the spermatid was formed and the blepharoplasts had begun their elongation. Where there are two nucleoli in a nucleus the nucleoli are much smaller than the single nucleolus which is sometimes found in each nucleus. The presence of sometimes one nucleolus, of two nucleoli, or in other cases of a constricting nucleolus, and the relative sizes of the nucleoli, all indicate that the nucleolus divides prior to each nuclear division.

The plastids were identified by their morphological characteristics in the prothallial cells, the antheridium initial, the wall cells, and in all spermatogenous cells until the spermatids were formed. After the formation of the spermatids the plastids were identified by their contained starch. This starch is abundant in the spermatids and mature spermatozoids. No starch was found in younger stages. The plastids of the spermatids were stretched to such an extent by enclosed starch that their boundaries could not be seen, even after staining.

A few rod-like or granular mitochondria are found in each prothallial cell. They are either grouped around the plastids or are scattered in the cytoplasm. In the division prior to the formation of the antheridium initial they are grouped about the poles of the spindle. The mitochondria are found in the antheridium initial and the antheridial walls, but are not as numerous there as in the spermatogenous tissue.

The mitochondria appear as short rods or granules with Champy-Kull's fixative and Altmann's stain. With Regaud's fixative they appear as long rods in some fixations and in other fixations have the appearance of a broken network. The mitochondria are very numerous in all stages of the spermatogenous tissue when both Champy-Kull's and Regaud's fixatives are used.

Soon after the nucleus begins to condense the mitochondria migrate from the peripheral cytoplasm toward the nucleus. They form a sheath around the partially condensed nucleus. The granules continue their migration as the spermatozoid matures. And in the fully developed spermatozoid they are found as two beaded rows along the inner surface of the coiled nucleus and extending as one row along that portion of the blepharoplast which protrudes beyond the nucleus.

It is a pleasure for the author at this time to acknowledge her indebtedness to Professor Duncan S. Johnson who suggested this problem and under whose direction this work was carried on. She would like also to thank Professor Conway Zirkle of Philadelphia for suggesting certain fixatives used and Professor Ralph E. Cleland of Baltimore for very helpful advice.

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DESCRIPTION OF PLATES

Figures 1–3 are photographs of living prothallia. Figures 4–63 are from paraffin sections of fixed prothallia. Figures 22–32 were fixed with Flemming's fixative and stained with Heidenhain's haematoxylin. Figures 33–40 were fixed with neutral formalin and Bouin's fluid and stained with Heidenhain's haematoxylin. Walls of cells and chromatin reticulum not stained. Figures 42–51, fixative, Champy-Kull's; stain, Altmann's. Figure 52, fixative, Merkel's; stain, Flemming's triple, walls in spermatogenous tissue not stained. Figures 53–63, fixative, Regaud's; stain, Heidenhain's haematoxylin.

General Explanation of Plates

All prothallia were cut parallel to the axis.

All figures were drawn with the aid of the Abbé camera lucida (Zeiss).

Magnifications (of figures as printed)

Figures 4–10 = × 450

Figures 11–13, 22 and 23 = × 650.

Figures 14–19 = × 270.
Figure 20 = × 170.
Figure 21 = × 90.
Figures 24–63 = × 2000

PLATE 23

Fig. 1. Antheridial plant in which the notch is formed.
Fig. 2. Male prothallium: antheridia cover whole surface except the three anterior rows of cells; some spermatozoids discharged.
Fig. 3. Culture of prothallia of *Polypodium polypodioides* including larger archegonial plants and smaller antheridial ones.

PLATE 24

Fig. 4. Vertical section of antheridium initial showing first mitosis.
Fig. 5. Three-celled archegonium.
Fig. 6. Young archegonium with two neck cells.
Fig. 7. First mitosis of central cell of archegonium.
Fig. 8. Ventral cell of archegonium in mitosis.
Fig. 9. Mature archegonium showing wall separating the neck canal cells; egg indented.
Fig. 10. Mature archegonium showing shallow egg cell: ventral cell; and two neck canal cell nuclei.
Fig. 11. Spermatozoid entering egg.
Fig. 12. Spermatozoid within the egg nucleus.
Fig. 13. Spermatozoid nucleus within egg nucleus, chromatin of former becoming reticulate.
Fig. 14. One-celled embryo.
Fig. 15. Two-celled embryo: wall slightly oblique to neck of archegonium.
Fig. 16. Four-celled embryo.
Fig. 17. Young embryo.
Fig. 18. Young embryo.
Fig. 19. Embryo showing primary organs differentiated; octant walls still distinct.
Fig. 20. Embryo slightly older than in fig. 19; octant wall distinct, but irregular.
Fig. 21. Older embryo: cotyledon initial; region of stem-growing point; foot; region of root-growing point.

PLATE 25

Fig. 22. Antheridium showing upper and lower wall cells and central cell.
Fig. 23. Antheridium with upper and lower wall cells, central cell and cap cell.
Fig. 24. Spermatid mother cell: two blepharoplasts.
Fig. 25. Spermatid mother cell in prophase: two blepharoplasts.
Fig. 26. Spermatid: blepharoplast in cytoplasm at some distance from nucleus.
Fig. 27. Spermatid: blepharoplast applied to nuclear membrane.
Fig. 28. Spermatid: blepharoplast beginning to elongate.
Fig. 29. Spermatid: nucleus beginning to condense; blepharoplast more than half around nucleus.
Fig. 30. Crescent shaped spermatid nucleus.
Fig. 31. Coiled spermatozoid: nucleus condensing; blepharoplast extending beyond nucleus.

Fig. 32. Spermatozoid found in neck of archegonium: 2.5 coils; cilia distinct.
Fig. 33. One-celled antheridium: central cell with two large irregular nucleoli.
Fig. 34. Two-celled antheridium: one nucleus with two nucleoli; one with constricting nucleolus.
Fig. 35. Four-celled antheridium: three nucleoli in section; two nuclei with one nucleolus each; one nucleus with constricting nucleolus.
Fig. 36. Eight-celled antheridium: four cells in section; three nuclei with two nucleoli; one nucleus with constricting nucleolus.
Fig. 37. Spermatid: blepharoplast near nucleus; two nucleoli.
Fig. 38. Spermatid: blepharoplast applied to nuclear membrane, one nucleolus.
Fig. 39. Spermatid: blepharoplast applied to nuclear membrane; two nucleoli.
Fig. 40. Spermatid: blepharoplast elongated; one nucleolus.
Fig. 41. Spermatid: blepharoplast elongated; two nucleoli.

PLATE 26

Fig. 42. Prothallial cell dividing to form anteridium initial: large plastids; mitochondria near poles of spindle.
Fig. 43. Antheridium showing central cell and two wall cells; plastids in wall cells large; plastids and mitochondria in the central cell.
Fig. 44. Two-celled antheridium: plastids in wall cells; plastids and mitochondria in spermatogenous tissue.
Fig. 45. Four-celled antheridium: plastids and mitochondria.
Fig. 46. Spermatid mother cell at metaphase: blepharoplasts at poles of spindle.
Fig. 47. Spermatid—blepharoplast applied to nuclear membrane; mitochondria in cytoplasm.
Fig. 48. Spermatid: nucleus beginning to flatten; blepharoplast half way around nucleus; mitochondria in cytoplasm.
Fig. 49. Spermatid: part of coiling nucleus; mitochondria on surface of nucleus and in nearby regions of cytoplasm.
Fig. 50. Mature spermatozoid: blepharoplast applied to anterior portion of nucleus and extending .5 of a coil beyond it; mitochondrial granules along inner surface of blepharoplast and nucleus.
Fig. 51. Mature spermatozoid: blepharoplast applied to anterior portion of nucleus and extending .5 of a coil beyond it; mitochondria as two rows along inner surface of nucleus and one row along blepharoplast.
Fig. 52. Mature spermatozoid with starch in protoplasmic vesicle.
Fig. 53. Antheridium initial: mitochondria in broken network.
Fig. 54. Spermatid: mitochondria as short rods.
Fig. 55. Spermatid: mitochondria as short and long rods.
Fig. 56. Spermatid: mitochondria as short rods; plastids.
Fig. 57. Spermatid: mitochondria as short and long rods.
Fig. 58. Spermatid: mitochondria forming broken network.
Fig. 59. Spermatid nucleus beginning to coil; mitochondria surrounding nucleus.
Fig. 60. Spermatid: nucleus coiling; mitochondria around nucleus.
Fig. 61. Mature spermatozoid: two rows of mitochondria along inner surface of nucleus; one row extending beyond it.
Fig. 62. Mature spermatozoid showing two rows of mitochondria.
Fig. 63. Mature spermatozoid showing two rows of mitochondria.

PLATE 23

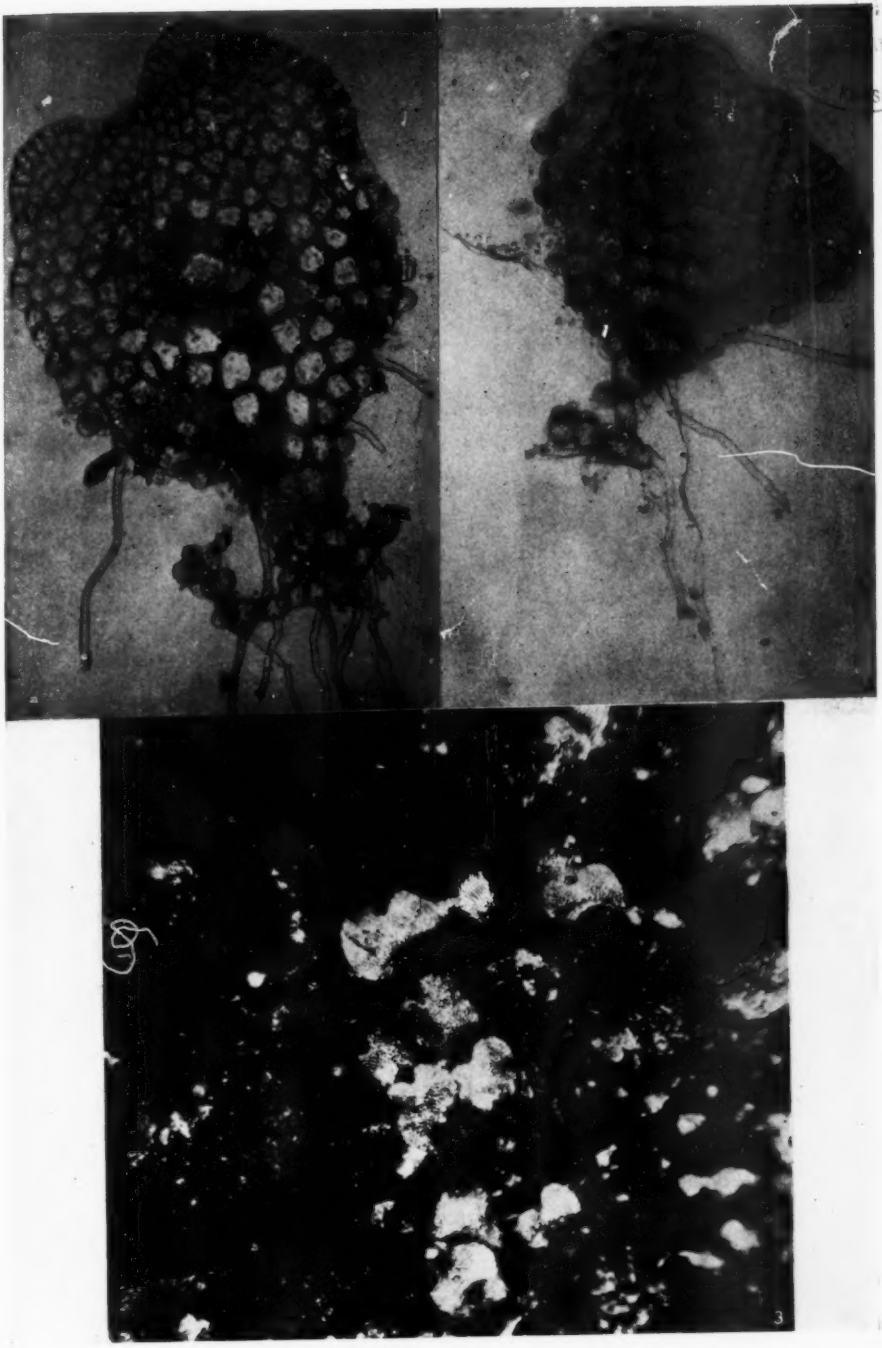

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PLATE 24

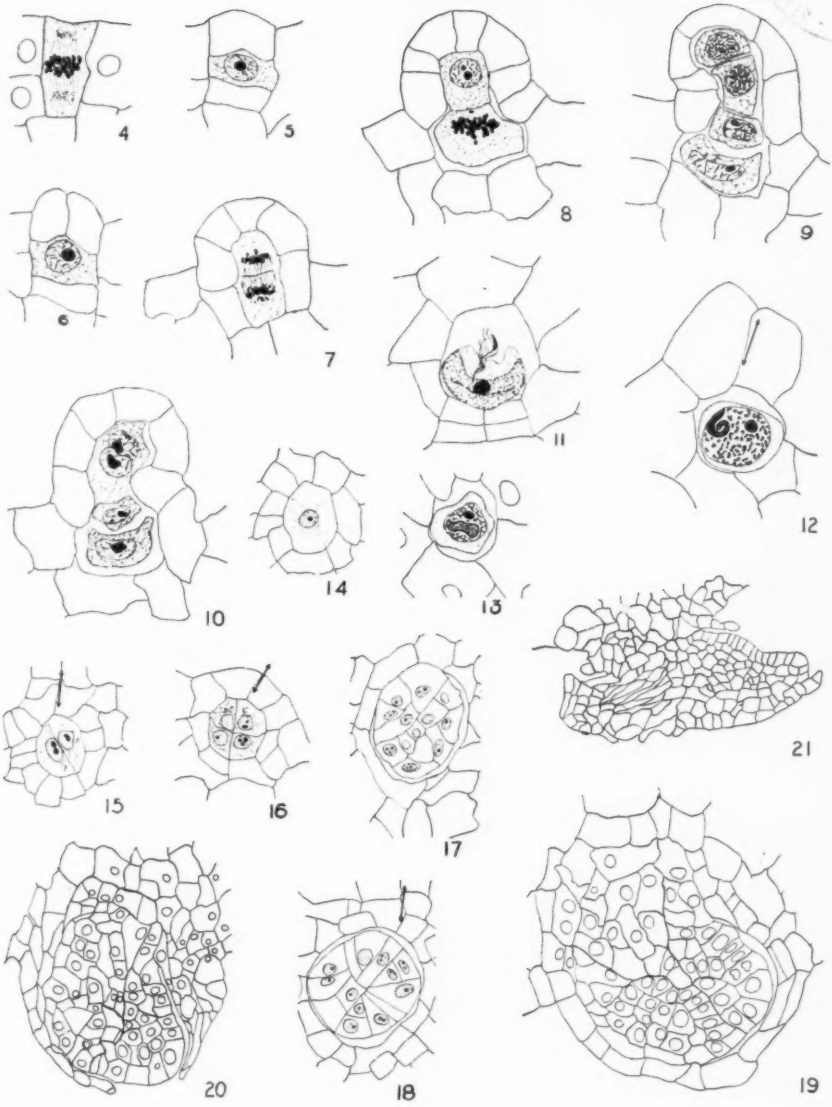

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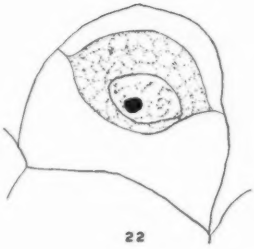
PLATE 25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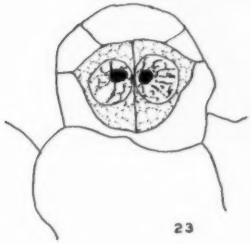
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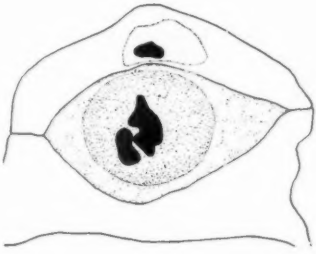
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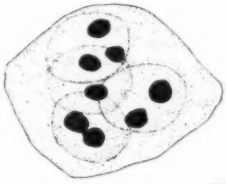
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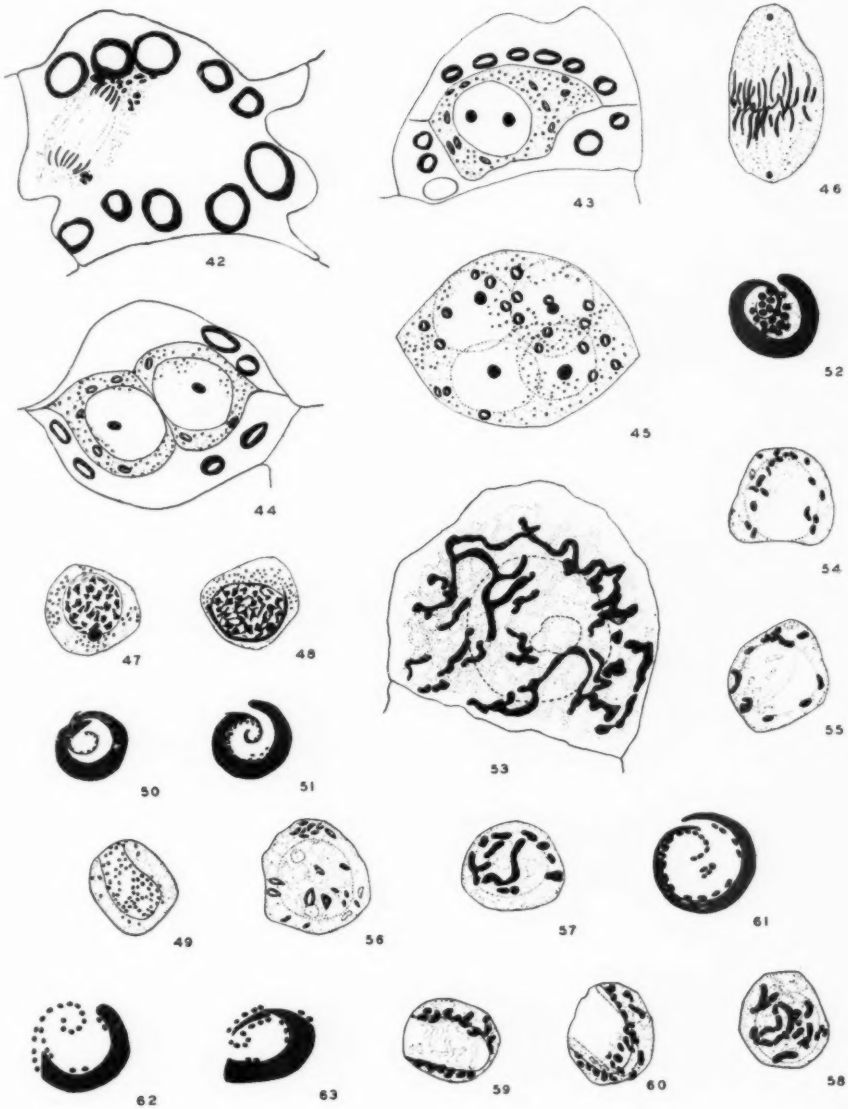
PLATE 26